THE USE OF ALGINATE AS A POTENTIAL CHONDROGENIC DIFFERENTIATION PROMOTER OF MESENCHYMAL STROMAL CELLS AND ITS APPLICATION

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ABSTRACT

Alginate culture system offers an ideal 3-Dimensional microenvironment for the re-differentiation of chondrocytes following its isolation from its environment and 2D culture expansion. It has also been shown that this material supports chondrogenesis for mesenchymal stromal cells *in vitro*. Bone marrow-derived mesenchymal stromal cells (MSCs) have been considered a potential cell source for cartilage repair. However, successful translation of such stem cell therapy into clinical trials using alginate as a carrier requires better understanding of the complex processes involved in the differentiation of MSCs. Changes in chondrogenic microenvironment may induce different outcomes during chondrogenesis, i.e. either stable chondrocyte-like phenotype or terminally differentiated hypertrophic chondrocytes, which may result in endochondral ossification formation following transplantation *in vivo*. Moreover there remains a question as to whether non-induced or lineage-committed MSCs should be applied for cartilage tissue engineering. Therefore the present thesis was conducted in 3 main studies to investigate the use of alginate as a scaffold or cell carrier in chondrogenic differentiation of mesenchymal stromal cells and cartilage repair.

In the first study, chondrogenic differentiated MSCs were characterized using morphological, biochemical and ultra-structural analyses. In the second study, expression of chondrogenic genes and adhesion molecules in alginate culture, pellet culture and 2D monolayer were compared using real-time RT-PCR. In the third study, alginate was transplanted with or without mesenchymal stromal cells in rabbit knee focal cartilage defects to determine whether chondrogenic MSCs, non-induced MSCs, or alginate alone will result in superior repair outcome in full thickness cartilage damage.

The results of the first part of the study demonstrated superior chondrogenic differentiation in the alginate group, indicated by the higher cell viability and production of chondrogenic markers of sulphated glycosaminoglycan and collagen type II as compared to monolayer or pellet cultures. Ultrastructural studies further revealed detailed cell structures, cell-matrix interactions and cell viability in 3D structures during chondrogenic differentiation. In the second part of the study, chondrogenic and hypertophic gene analysis demonstrated the hypertrophic nature of chondrogenesis in 2D monolayer and 3D pellet culture while non-hypertrophic features were observed in alginate. In the third study, the application of alginate in cartilage repair either as a carrier for undifferentiated MSCs or as a scaffold for chondrogenic differentiation of MSCs before being transplanted, improved the cartilage repair outcome in rabbit knees. However there were no differences observed in the outcome following transplantation of alginate alone in cartilage defects compared to the non-treated knee.

In conclusion, the present thesis suggests that alginate may prove to be a potential biomaterial that provides a favourable environment for chondrogenic differentiation of MSCs *in vitro* and *in vivo*, thereby proving itself as a potential candidate scaffold/cell carrier for clinical application involving the repair of damaged cartilage.

ABSTRAK

Sistem kultur alginat menawarkan persekitaran mikro 3-Dimensi yang sesuai untuk pembezaan kondrosit setelah melalui perkembangan kultur 2D. Kaedah in terbukti

menyokong proses kondrogenesis bagi sel-sel stromal mesenkimal secara *in vitro*. Sel-sel mesenkimal stromal (MSC) yang diperoleh daripada tulang sum-sum merupakan sel punca yang berpotensi untuk pembaikan tisu rawan. Walau bagaimanapun, kejayaan ujian klinikal terapi sel punca di dalam persekitaran mikro alginate memerlukan pemahaman yang lebih baik kerana pembezaan MSC melibatkan proses yang kompleks. Perubahan dalam persekitaran mikro kondrogenik boleh memberikan hasil yang berbeza semasa proses kondrogenesis. Ia boleh menghasilkan kondrosit yang mempunyai fenotip yang stabil ataupun kondrosit hipertrofi yang mengalami pembezan secara terminal di mana ia menyebabkan pembentukan osifikasi endokondral selepas transplantasi secara *in vivo*. Tambahan pula masih timbul persoalan sama ada 'lineage-commited' MSCs boleh digunakan untuk kejuruteraan tisu tulang rawan. Oleh itu tesis ini mengandungi 3 kajian utama yang telah dijalankan untuk menyiasat penggunaan alginat sebagai konstruk 3-Dimensi yang sesuai dalam proses pembezaan kondrogenik sel-sel stromal mesenkimal dan pembaikan tisu rawan.

Dalam kajian pertama, MSC yang melalui pembezaan secara kondrogenik telah diklasifikasikan dengan menggunakan analisa morfologi , biokimia dan ultra- struktur. Dalam kajian kedua , ekspresi gen kondrogenik dan molekul lekatan di dalam kultur alginate telah dibandingkan dengan kultur pelet dan lapisan mono 2D menggunakan kaedah 'real time RT- PCR'. Dalam kajian terakhir, alginat yang mengundungi sel dan tanpa sel, ditransplantasi ke dalam sendi lutut arnab yang mengalami kerosakkan. Ini adalah untuk menentukan sama ada hanya MSC kondrogenik yang tidak terinduksi, atau hanya alginat yang akan mempengaruhi proses pembaikan kerosakkan tisu rawan.

Keputusan kajian menunjukkan, kumpulan alginat menyokong proses pembezaan kondrogenik. Ini dibuktikan melalui proliferasi sel yang lebih tinggi, pengeluaran penanda kondrogenik daripada glikosaminoglikan sulfat dan kolagen jenis II dalam alginat jika dibangdingkan dengan lapisan mono atau kultur pelet. Kajian Ultrastruktur mendedahkan lagi struktur terperinci sel, interaksi sel -matriks dan proliferasi sel dalam struktur 3D semasa pembezaan kondrogenik. Dalam bahagian kedua kajian ini, Kultur lapisan mono 2D dan kultur pelet 3D yang melalui proses kondrogenesis menunjukkan ciri-ciri hipertrofik. Manakala ciri-ciri bukan hipertrofik diperhatikan dalam alginat yang melalui proses kondrogenesis. Dalam kajian terakhir, penggunaan alginat sebagai konstruk 3D yang menyokong pembezaan kondrogenik daripada MSC sebelum ditransplantsi, menunjukkan hasil yang baik dalam pembaikan tulang rawan di dalam sendi lutut arnab. Walau bagaimanapun tiada perbezaan yang ketara dapat diperhatikan dalam transplantasi yang hanya menggunakan alginat jika dibandingkan dengan lutut yang tidak dirawat .

Kesimpulannya, tesis ini menunjukkan alginat berpotensi untuk menjadi satu biobahan yang mampu menyediakan persekitaran yang kondusif bagi pembezaan kondrogenik daripada MSC secara *in vitro* dan *in vivo*. Dengan ini, terbukti bahawa alginate merupakan konstruk 3D yang menyokong perkembangan sel punca seperti MSCs dan sekaligus berpotensi dalam aplikasi klinikal yang melibatkan pembaikan tulang rawan yang rosak.

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LIST OF SYMBOLS AND ABBREVIATIONS

3D- 3 dimensional

ACAN- Aggrecan

ACI- Autologous chondrocyte implantation

ACS- Adult stem cells

ACT- Autologous chondrocyte transplantation

Al^{3+}- Aluminium

APC- Allophycocyanin

Ba^{2+}- Barium

BMPs- Bone morphogenic growth factors

bp- base pair

BSA- Bovine serum albumin

Ca^{2+}- Calcium

CD105- Cluster of Differentiation (Endoglin, accessory receptor for TGFβ)

CD29- Cluster of Differentiation (Integrin β1)

CD34- Cluster of Differentiation (haematopoietic progenitor cell marker)

CD 44- Hyaloronic receptor(H-CAM)

CD 45- Leuckocyte common antigen Ly-5

CD73- Ecto-5' nucleotidase

CD90-Thy-1

cDNA- Complementary deoxyribonucleic acid

CMSC- Chondrogenic mesenchymal stromal cells

Col I- Type I Collagen

Col II- Type II Collagen α1

Col X- Type X Collagen

CPD- Critical Point Dryer

Cq-Quantification cycle

d- Day

DAB- 3, 3’ Diaminobenzidine

DAPI- 4', 6-diamido- 2-phenylindole

DDSA- Dodecenyl Succinic Anhydride

ddw- Double distilled water

DMEM-HG- Dubleco modified eagle medium high glucose

DMEM-LG- Dubleco modified eagle medium low glucose

DMP-30- Dimethylaminomethyl phenol

dw- Distilled water

ECM- Extracellular matrix

EthD- Ethidium Homodimer

FDA- Food and Drug Administration

Fe^{+3}- Iron

FGF- Fibroblast growth factor

FITC- Fluorescein isothyocyanate

FSC- Forward-light scatter

GAG - Glycosaminoglycans

GAPDH- Glyceraldehyde-3-phosphate

HA- Hyaloronic acid

HBP- Heparin-binding peptide

HMSC- Human bone marrow derived stromal cells

HRP- Horseradish peroxide

IGF- Insulin-like growth factor

Ihh- Indian hedgehog

ITS- Insulin Trasnferrin Selenium

mAbs- Monoclonal antibodies

MAPK- Mitogen-activated protein kinases

MMP- Matrix metalloproteinase

mRNA- Messenger ribonucleic acid

MSC- Mesencymal stromal cells

MW- Molecular weight

Ncad- N Cadherin

NCAM- Neural cell adhesion molecule

OA- Osteoarthritis

PE- Phycoerythrin

PHrP- Parathyroid Hormone-Related Protein

RGD peptide -Arg-Gly-Asp - Arginylglycylaspartic acid

rRNA- Ribosomal RNA

RT-PCR- Reverse Transcriptase Polymerase Chain Reaction

RT- Room temperature

RunX2- Runt family transcription factor 2

Shh- Sonic Hedgehog

Smad- small 'mothers against' decapentaplegic (intracellular proteins)

Sox - Sex-determining region Y-type high motility group box

Sr^{2+}- Strontium

SSC- Side-light scatter

TAE-Tris-acetate-EDTA

TBS- Tris- Buffered Saline

TGFβ-Transforming growth factors β

VEGF-Vascular endothelial growth factor

W/V- Weight per volume

Wnt- Wingless related protein

Zn^{2+}- Zinc

LIST OF APPENDICES

1 CHAPTER 1

1.1 General Introduction

Articular cartilage being the load-bearing tissue of the joint has limited repair and regeneration potential as the result of its poor tissue vascularity. Hence, once damaged, long-term disability can be expected in patients. For many years, this issue remains to be an unsolved clinical challenge in the field of orthopaedics. Traditional treatment modalities intended to regenerate cartilage, such as abrasionplasty, subchondral drilling and microfracture only provides short-term benefits at best (Hunziker E.B. *et al.* 2002; Gomoll A.H. *et al*. 2012). To overcome this, tissue engineering approaches using cells seeded in scaffolds have been introduced. This treatment option presents itself as a viable alternative to introduce techniques that promises better outcome. However, how much better is this method as compared to other more conventional techniques? In most studies, results of using these constructs to treat damaged cartilage has shown to be promising (Løken S. *et al.* 2008; Lin L.C. *et al*. 2012; Jang K.M. *et al.* 2013). In a number of systematic reviews, it has been shown that the use of cell therapy is somewhat superior to that of many known techniques (Naveen S. *et al*. 2012, Gopal K. *et al.* 2014). Generally, cell therapy for cartilage has been confined to the use of cultured articular chondrocytes, such as autologous chondrocyte implantation (ACI), which is an FDA approved technique. However, mesenchymal stem cells (MSCs), has been considered to be an alternative cell source to native chondrocytes because such cells may be made available from different tissues such as adipose and bone marrow. In addition to MSCs possessing potent anti-inflammatory and immunomodulatory effect, it has been postulated that good or excellent outcomes observations can be expected as the result of either direct cell-cell interaction or secretion of different factors (Caplan A.I. *et al*. 2006; Iyer S.S. *et al*. 2008; Oh J.Y. *et al*. 2008).

Various *in vivo* experiments have described superior repair outcomes when using MSCs with supportive scaffolds in treating cartilage defects (Wakitani S. *et al*. 2002; Yan H. *et al*. 2007; Qi Y. *et al*. 2012; Zhao Q. *et al*. 2013). Naturally derived biomaterials such as agarose, hyaluronic acid, chitosan, collagen, gelatine, and alginate have been used in cartilage tissue engineering. The main advantages of using such scaffolds are their biodegradability and biocompatibility properties (Yoon D.M. *et al*. 2006). Among these scaffolds, only hyaluronic acid and collagen have been used in clinical studies using MSCs (Table 1.1). Alginate is a well characterized biomaterial that has been largely described in literatures (Draget K.I. *et al*. 1997; Sun J. *et al*. 2013). Its use has included applications such as wound dressings (Clark M. *et al*. 2012) and drug delivery systems (Tønnesen H.H. *et al*. 2002). More recently, alginate has been used as a carrier for chondrocyte in clinical trials for treatment of cartilage defects (Selmi T. A. S. *et al*. 2008).

Despite the variety of clinical application of alginate, it has not been used in clinical applications of cartilage repair using MSCs. The reasons for this appear to be undetermined since the biomechanical properties or the ability for the material to allow cell attachment is not the issues of this material. However, there are arguments that suggest that alginate may not possess the necessary biomechanical characteristics that can allow it to perform as good as native cartilage tissue. Hence, in many studies alginate is used mainly as a delivery vehicle rather than a scaffold for cartilage repair purposes (Diduch D.R., *et al.* 2000; Tay L.X., *et al.* 2012). Moreover the mechanical properties of alginate can be enhanced by increasing its concentration and/or gluronic acid contents in its molecular structure (Enobakhare B.O *et al.* 2006). Cell adhesion properties can also be improved using RGD peptide modification into the pourus alginate scaffolds(Re'em T. *et al.* 2010) or alginate gel-microsphers(Sun J. *et al.* 2013). However, it has become apparent that the use of alginate may result in differing

outcomes when used in experiments; resulting in either the formation of the preferred cartilage-like tissues or, the hypertrophic-like cartilage tissues that is less desirable.

The review of present available literatures demonstrates that studies relating to the chondrogenic differentiation of MSCs in alginate has been reported extensively (Xu J.P. *et al*. 2008; Duggal S. *et al*. 2009; Herlofsen S.R. *et al*. 2011, Herlofsen S.R. *et al*. 2013). It is interesting to note that the use of the same biomaterial as a 3D matrix for chondrogenic differentiation of MSCs produces varying degrees chondrogenic differentiation. One group of researchers described the chondrogenic differentiated MSCs in alginate as an articular cartilage model (Yang I.H. *et al*. 2004; Andriamanalijaona R. *et al*. 2008), while others have reported that when cultured *in vitro*, the expression of hypertrophic markers can be expected (Ma H.L. *et al*. 2003; Steinert A. *et al*. 2003; Ichinose S. *et al*. 2005; Xu J. *et al*. 2008; Bian L. *et al*. 2011).

It is suggested that the source of cell itself, such as bone marrow derived MSCs may have a role in the observed hypertrophic state although further investigation relating to this needs to be substantiated (Brian Johnstone *et al*. 2013). In other studies, it has also been postulated that the surrounding microenvironment may be the determining factor for the different fates observed within the same cell source. It is suggested that the reason for this may be due to the epigenetic effect being exerted during the chondrogenic differentiation process. This process appears to be similar to that observed during the normal development of cartilage. Epigenetics mechanisms are stable changes in gene expression, in which histone modification and DNA methylation influence the chromatin structure (Furumatsu T. et al. 2010). These changes although heritable do not alter the genetic messages (Jaenish R.et al.2003)

During the limb budding stages at the embryonic development of vertebrate, the initial population of MSCs undergo chondrogenesis resulting in two distinct cell fates. One

group of cells is directed to form articular cartilage at the joint surfaces, arrest maturity before terminal differentiation to a hypertrophic chondrocyte and maintain their chondrogenic phenotype throughout life unless in pathological conditions such as osteoarthritis (OA) (Drissi H. *et al*. 2005, Goldring M.B., *et al.,* 2007). The second group of cells destined to form the shaft of the limb, chondrogenic cells undergo maturation, hypertrophy, apoptosis, vascular invasion and finally replaced by bone. This processes appears to undermine the mechanisms for long bone growth in epiphyseal growth plate (Drissi H. *et al*. 2005; Pacifici M. *et al.* 2005).

In vitro chondrogenic differentiation models usually apply high density of cells in the form of pellet or aggregates or a combination of cells and biomaterials to provide a 3D structure for maximum cell-cell and cell-matrix interactions (Penick K.J. *et al*. 2005). In addition, stimulating factors such as transforming growth factor-β (TGFβ), Insulin like growth factors and bone morphogenic proteins (BMPs) are needed to induce chondrogenesis (Seo S. *et al*. 2011). However for its application in articular cartilage tissue engineering, the differentiated cells should produce tissues that mimics articular cartilage model and arrest maturity before terminal hypertrophic differentiation occurs. This is done in order to provide a natural glistening and smooth surface needed on cartilage (Rubin R. *et al*. 2011) for normal joint movement after transplantation in defective area.

In this study in order to further explore the chondrogenic differentiation potential of MSCs in alginate beads *in vitro*, alginate is compared to 2D monolayer and the most commonly used 3D chondrogenic model i.e., pellet culture, which possess a hypertrophic nature for chondrogenic differentiation (Yang I.H. *et al*. 2004; Liu T.M. *et al*. 2007; Uesatoll R. *et al*. 2008; Pelttari K. *et al*. 2010). In order to study the cartilage repair, MSCs or chondrogenic MSCs (CMSC) loaded in alginate or alginate beads without cells were then transplanted in the defective area of rabbit knee cartilage.

1.2 Rationale for conducting this study

Chondrocytes in articular cartilage maintain their chondrogenic morphology and physiology which, unlike chondrocytes at the epiphyseal plate do not undergo hypertrophy at early maturation. In pathological conditions such as osteoarthritis, changes are observed and ultimately results in poor joint function. As such, the use of engineered cartilage that results in these unwanted outcomes would be deemed inappropriate for use to repair damaged cartilage. To overcome this problem, many studies have looked into improving the biomaterials that avoids such outcomes.

Alginate, although have been selected as a potential material, have not been researched into great depth specifically in the areas of cartilage repair or regeneration in clinical applications. In previous studies, there have been controversies between those who support and refute its usefulness for this application. Among which, several researchers have indicated that hypertrophic markers during chondrogenic differentiation are observed and therefore may be detrimental to cartilage repair outcomes, while others have demonstrated otherwise. Considering that there is likelihood that alginate, being a biomaterial that has been shown to be useful for chondrogenesis previously, and that it serves as a potential carrier or scaffold for tissue engineering; it is only appropriate that a complete study looking into its potential be undertaken. Thus, the present thesis encompasses studies that looks into the potential of alginate as a promoter of chondrogenesis in primary mesenchymal stromal cells, and as such will express the appropriate functions and phenotypic expressions that would eventually be useful for repairing damaged cartilage.

1.3 Review of the literature

1.3.1 Alginate

Alginate has been used for more than one century in the food industry, textile printing, pharmaceutical and several medical uses including immobilized biocatalyst (McHugh *et al*. 2003). Alginate is a natural and un-branched polysaccharide commercially derived from brown alga or biologically synthesized from bacteria (Sabra W. *et al*. 2001; Hay I.D. *et al*. 2010). In the cell wall of brown sea weed, alginate may be present as a sodium, calcium or magnesium salts of alginic acid, since the calcium and magnesium salts are insoluble in water only sodium alginate is the goal of extraction (McHugh *et al*. 2003).

Alginate molecule is made of co-polymer components of mannuronate-guluronate (MG) containing (1,4)-linked β- D-mannuronate (M) and α -L-guluronate (G) (Fig. 1-1) The G content is varied between 40% to 70% and the sequence of monomers are also different in different industrial products (Andersen T. *et al*. 2012). Alginate can be manufactured with variable content of G and M blocks. G blocks are the main part for crosslinking, higher G content results a stronger gel (McHugh *et al*. 2003). Concentration of alginate itself and ionic cross linkers were also contributed to the gel strength (Martinsen A. *et al*. 1989; LeRoux M.A. *et al*. 2000; Enobakhare B.O. *et al*. 2006).

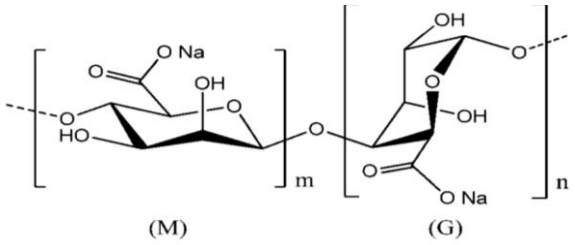


Figure 1-1 chemical structure of alginate containing G and M blocks.

Tokarev A. *et al*. (2012)

Different applications of alginate are based on their heterogeneous chemical properties. One of these properties is forming gel due to their ionic crosslinks (McHugh *et al*. 2003). Alginate can be cross-linked through ionic cross-linkers such as Ca^{2+}, Ba^{2+}, Sr^{2+},Al^{3+},Zn^{2+}, Fe^{+3} (Machida-Sano I. *et al*. 2012; Olukman M. *et al*. 2012). Among these ions, calcium is a more popular cross linker for cell delivery purpose because of its non-toxic nature (Nussinovitch A. *et al*. 2010).

Cross-linking can take place with external exposure by dripping into a calcium chloride solution or internal release of calcium in alginate. When Na- alginate is dropped into a calcium solution, calcium ions bind to poly G segments (G-blocks) in different alginate chains and produce more cross-links (Fig. 1-2). Covalently cross linking of metacrylated alginate is formed using carbdamiide chemistry in the presence of a photo initiator and UV light (Chou A.I. *et al*. 2009; Jeon O. *et al*. 2009). Using this method, bioactive peptides such as RGD (arginine- glycine- asparagine) and HBP (Heparin - binding peptide) covalently attach to alginate to facilitate cell attachment through integrins or non-integrin adhesions (Sapir Y. *et al*. 2011).

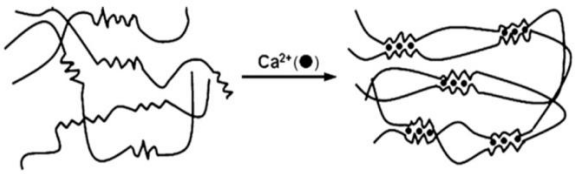


Figure 1-2 Ionic cross-linking in alginate

(Lee K.Y. *et al*. 2007)

Alginate has been used in a variety of physical forms in biomedical applications including, gel, fibres, beads, and microcapsules (Thu B. *et al*. 1996; Trouche E. *et al*. 2008; Tabata Y. *et al*. 2014) in which the resulted ionically cross-linked alginate microbeads are wrapped in another layer of poly (L-lysine) to reduce permeability and

increase stability. Such microcapsules were used for immunoisolation of transplanted tissues such as pancreases (De Vos P. *et al*. 2006). Porous alginate/foam/ sponge (AlgiMatrix®) more commonly applies for studying of soft tissues such as liver cells (Glicklis R. *et al*. 2000; Glicklis R. *et al*. 2004; Ramasamy T.S. *et al*. 2013; Capone S.H. *et al*. 2013) give them a suitable environment to have cell-cell interactions and form aggregates or spheroids in a predefined open space, which facilitate exchange of nutrient and gases and allow vascularization *in vivo*.

In vivo degradation of alginate can occur to the process of ion exchanges of calcium with sodium. In addition, reactive oxygen species (ROS) can also affect alginate degradation during sterilization with gamma irradiation (Andersen T. *et al*. 2012).

1.3.2 Alginate in clinical application

Alginate is approved by the US Food and Drug Administration (FDA) for use in humans as a wound dresser and in the food industry. However the usage of alginate in biomedical/pharmaceutical including tissue engineering applications must first be specifically approved by a national governing body such as the FDA (Andersen T. *et al*. 2012). For this purpose the product should be tested in pre-clinical trials including *in vitro* and *in vivo* experiments to establish the characteristics and safety of the biomaterial, which will include biocompatibility and biosafety of the treatment (Lee M.H., *et al.* 2009). The clinical trials initially have three Phases (0, I, II) to evaluate pharmacodynamics, safety, and pharmacokinetics within smaller groups of patients and phase III including a larger population of people involved in randomized trial to verify effectiveness (Andersen T. *et al*. 2012). Even after a product is marketed, FDA continues its supervision in different ways, such as inspections, post approval(Phase4) studies, and surveillance of the side effects reported to FDA (Lee M.H., et al. 2013).

As mentioned earlier, the issues shrouding the use of alginate in clinical applications remains unclear however, it is clear that further studies would be needed in order to satisfy the needs of regulatory bodies such as FDA before alginate can be used clinically.

Having said this, alginate has been used in several clinical trials such as the encapsulation of pancreatic islets for treatment of type I diabetes patients. Others have also included trials such as a bone substitute with combination of collagen and demineralized bone matrix to treat spine fusion and as a biodegradable bone void filler. Its combination with propylene glycol and amelogenin (enamel matrix protein) is used to stabilize teeth and to induce tissue regeneration in periodontal diseases. It also has been used as a suture sealant during lung resection in cancer treatment. Injection of alginate chondrocytes was carried out in the submucosa of bladder in paediatric patients at the vesicouretric junction to prevent ureteral reflux (Leonard M. P. 2002; Andersen T. *et al*. 2012, U.S. National Institutes of Health 2013). In recently published clinical trials, sodium alginate have been orally prescribed to treat patients with non-erosive reflux disease (Chiu C.T. *et al*. 2013), and finally a commercial form of alginate-agarose combination (CARTIPATCH®) has been used as a carrier for chondrocytes in a clinical trial to treat osteochondral/chondral lesions of the knee (Selmi T.A.S. *et al*. 2008).

1.4 Articular cartilage injury

Damage to the cartilage tissue as the result of trauma or disease conditions are becoming increasingly common. The poor vascular property of cartilage tissue makes healing extremely difficult especially in the presence of full thickness articular cartilage defects. Different surgical treatments have been developed to deal with cartilage poor repair quality; some of these methods are based on marrow stimulating techniques, such

as subchondral drilling, abrasion, or microfractures. Other techniques such as ostochondral graft, mosaicplasty, periosteal and perichondrial grafts as replacement techniques have been used more commonly (Memon A.R. *et al*. 2012). A technique such as osteotomy fixes the mal-alignment of the joint in order to remove the extra load from the defected area are even added to improve clinical outcomes (Nelson L. *et al*. 2010). However the long term results of different surgical treatments for cartilage injury are disappointing and if they fail, the salvage and invasive methods employed such as arthrodesis or arthroplasty provide less acceptable outcomes for patients (Panagiotopoulos E. *et al*. 2006; Haslam P. *et al*. 2007). Therefore cell-therapy as another alternative treatment of cartilage injury has been applied which promises superior outcomes to that of these methods.

1.4.1 Cell Therapy

Cellular therapy has been considered a successful treatment modality for the repair of damaged articular cartilage, producing superior tissue repair quality as compared with the standard surgical approach (Brittberg M. 2010). The first clinical trial reporting the efficacy of autologous chondrocyte implantation/transplantation (ACT/ACI), the conventional form of cellular therapy, was published in 1994 (Brittberg M. *et al*. 1994) after its successful trials in animal studies in the preceding years (Grande D.A. *et al*. 1989; Wakitani S. *et al*. 1989).

ACI has been studied in many clinical trials with good outcomes in the majority of cases (Brittberg M. *et al*. 1994; Peterson L. *et al*. 2000; Bartlett W. *et al*. 2005; Manfedini M. *et al*. 2007). For example, in one study (Cole B.J *et al*. 2012) 85% of patients with osteochondritis dissecans, who already had experienced other methods unsuccessfully, had functional improvement and reduction of pain after ACI. However

first and second generation of ACI that involves the use of periosteal flap or collagen membrane, are technically difficult as many surgeons have found that suturing the flaps to the surrounding cartilage and in some areas such as osteochondral junctions or irregular defects proves to be a difficult task. Moreover the cell distribution in these methods is not uniform (Goyal D. *et al*. 2013). In order to overcome these problem, third generation of ACI using seeded chondrocyte on an extracellular matrix or MACI (Matrix associated autologous chondrocyte implantation/membrane seeded chondrocytes (Goyal D. *et al*. 2013) have been introduced.

Despite a successful historical performance for more than 18 years, the use of ACI has not been without limitations. Its recognized shortcomings include donor-site morbidity, limited supply of chondrocytes, cellular dedifferentiation into fibroblast-like phenotype when cultivated *in vitro*, and inability to maintain good tissue repair in the long term (Brittberg M. 1999; Hunziker E.B. *et al*. 2002). It has been speculated that the use of an alternative cell source such as mesenchymal stem cells (MSCs) might overcome some of these issues.

MSCs were first used for cartilage repair in humans as an endogenous cell therapy (Pridie K.H. 1959; Kim S.H. *et al*. 2012) for the treatment of cartilage damage in conjunction with other surgical techniques which recruit MSCs from bone marrow; techniques such as microfracture, and most recently a combination of microfracture and scaffold. However inadequate number of MSCs retrieved from bone marrow for the repaired site in such techniques reduces their efficacy and as a result the repaired tissue represents a fibrocartilage with inferior biomechanical properties as compared to hyaline cartilage (Nishimori M. *et al*. 2006). Therefore exogenous source of MSCs has been considered as mesenchymal stem cells may be harvested from many potential donor sites including bone marrow, adipose tissue (Im G-II *et al*. 2005; Kim D.H. *et al*. 2013), trabecular bone, periosteum, synovium and dental pulp (Pelttari K. *et al*. 2008;

Mobasheri A. *et al*. 2009). In addition, with a high proliferation capacity, and owing to their multipotency, these cells can conveniently be manipulated *in vitro* to differentiate into chondrocytes for subsequent use in cartilage regeneration (Darwin J. *et al*. 1998; Caplan 2007; Karp J.M. *et al*. 2009).

There have been many previous reports involving *in vivo* experiments describing good repair outcomes after transplantation of MSCs in cartilage defects. Wakitani *et al.* in 1994 were among the first research groups to report the successful transplantation of bone marrow–derived MSCs in osteochondral defects in rabbit models. Other researchers have also studied the application of allogeneic or autologous bone marrow–derived MSCs using different scaffolds with or without the addition of growth factors to treat cartilage defects in various animal models (Gao J. *et al*. 2002; Murphy J.M. *et al*. 2003) (Fig. 1-3).

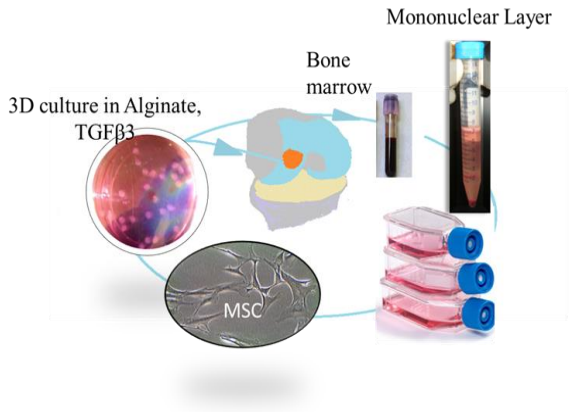


Figure 1-3 Cartilage tissue engineering, using cells, scaffold and growth factor

1.4.2 Scaffolds in cartilage tissue engineering

Different strategies being used to engineer cartilage in addition to application of proper cell source and growth factors strongly depends on the selection of the scaffolds. In cartilage tissue engineering scaffolds not only provide a convenient method for delivering cells into focal defect sites, but also provide structural support to the

construct and induce cartilage matrix formation within the defective sites (Vinatier C. *et al*. 2009; Zippel N. *et al*. 2010). Scaffolds can be produced from different type of materials including polymers (Yoon D.M. *et al*. 2006) (Fig. 1-4).

Naturally derived polymers are classified into two groups, polysaccharides and polypeptides (Ma P. X. 2004; Yoon D.M. 2006). Different naturally derived polymers have been applied in cartilage tissue engineering including: Agarose (Yin Z. *et al.* 2014) alginate (Herlofsen S.R. *et al.* 2011), hyaluronic acid (Solchaga L.A. *et al.* 2005), chitosan (Ragetly G.R. *et al.* 2010; Suh J.K. *et al*. 2000), collagen (Deponti D. *et al*. 2013), chondroitin sulphate (Guo Y. *et al*. 2012), Cellulose (Pulkkinen H. *et al*. 2006) Gelatin (Schagemann J.C.,*et al*. 2009), fibrin (Eyrich D. 2006), and silk (Seda Tigli R. *et al*. 2009).

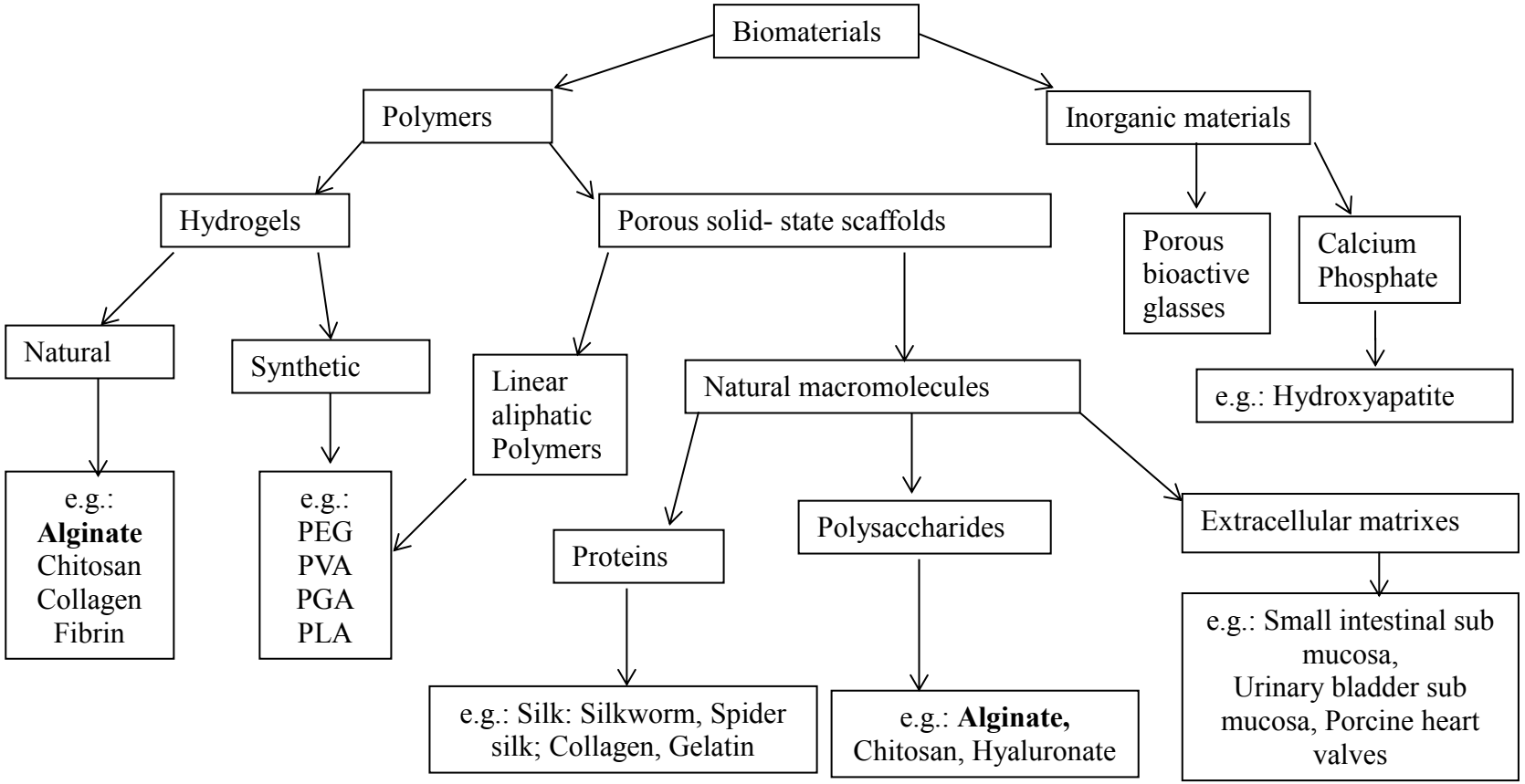


Figure 1-4 Biomaterials for tissue engineering

Polymers being used in tissue engineering in form of hydrogels or porous solid –state scaffolds . Inorganic materials in form of Porous bioactive glasses or calcium phosphate. Alginate as a naturally derived polymer is used as a hydrogel or solid- state porous scaffold in tissue engineering. Adapted from Ma P.X. 2004

Among the described naturally derived polymers, only hyaluronic acid and collagen are being used as a cell vehicle or scaffold in cartilage regeneration clinical trials using MSCs (Table 1-1).

Table 1-1 Commercial scaffolds using in clinical trials for cartilage repair

Scaffold	Natural		Synthetic	Cell
	Protein	Polysaccharide		
MACI®	Collagen I & III membrane	-	-	Chondrocyte
Maix®	Collagen I & III membrane	-	-	Chondrocyte
Chondro-gide®	Collagen I & III membrane	-	-	Chondrocyte
Atelocollagen®	Collagen I-gel	-	-	Chondrocyte, MSCs
Hyalograft®	-	Hyaluronic acid	-	Chondrocyte
HYAFF11®	-	Hyaluronic acid	-	Chondrocyte, MSCs
Bio-Seed-C®	-	-	PGA-PLA-polydioxanone	Seeded chondrocytes within a fibrin gel
CARTIPATCH®	-	Alginate-Agarose	-	Chondrocyte

Adapted from: Selmi T. A. S. 2008; Vinatier C. 2009; Iwasa J. 2009

There are strict criteria to be met for a biological product to be approved with FDA for clinical application (US Food and Drug Administration 2011, US Food and Drug Administration 2014). Tissue engineering products are not described exclusively by components alone since cell–scaffold interactions also influence the characteristics of the final product. In evaluating cell–scaffold products, individual components before the assembly as well as the whole product after assembly should be evaluated with a variety of tests. Figure 1-5 summarizes the main safety and characterization of two main components of cell and scaffold that should be considered for tissue engineering.

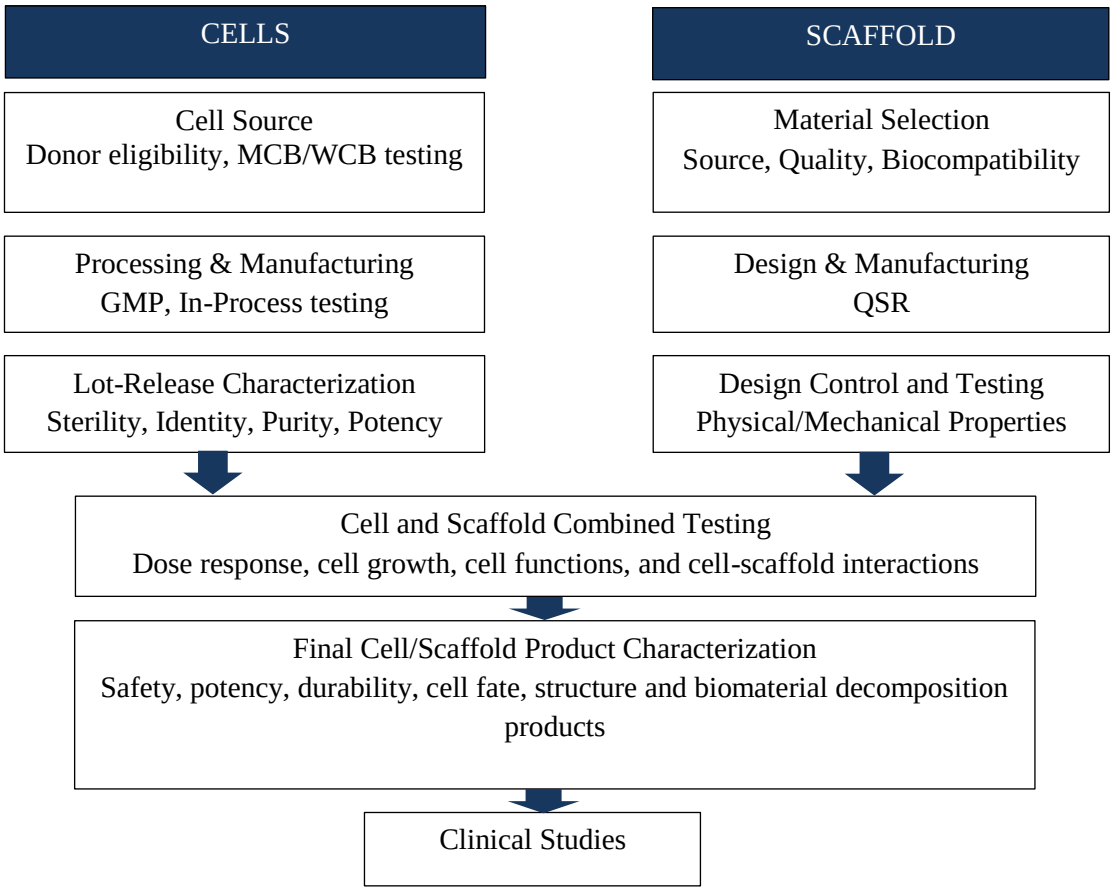


Figure 1-5 Safety and effectiveness measures of a cell–scaffold combination to be used in a clinical trials GMP, good manufacturing practice; MCB, master cell bank; QSR, quality systems regulation; WCB, working cell bank (Lee M.H., 2009).

1.4.3 Regulation of chondrogenic differentiation

MSCs can be induced along a chondrogenic lineage using biochemical and biophysical factors. (Bahuleyan B. *et al.* 2009). It has been demonstrated that mechanical loading exerts an important effect on the chondrogenic differentiation of MSCs during the development of the musculoskeletal system as well as during cartilage repair using transplanted MSCs as shown in several animal studies (Bahuleyan B., *et al.* 2009, O'Conor C.J., *et al.* 2013) or *in vitro* chondrogenic differentiation (Henrionnet *et al.* 2012)

Chondrogenic differentiation is initiated in the embryo with aggregation of mesenchymal stem cells in the incipient limb site in a process called condensation. Cell-cell communications seems to play an important role at this stage as it was shown by the expression of adhesion molecules of N-cadherin and N-CAM1 and gap junctions; i.e. during the prechondrogenic phase (Widelitz R.B *et al.* 1993, Delise A.M., *et al.*2002). Multiple growth factors and morphogens such as TGFβ and FGF regulate the initiation and maintenance of chondrogenesis. Having bound to their serine/threonine kinase receptors, TGFβs stimulate downstream signalling pathways through SMAD proteins or MAPK signal transducers (Wang W *et al.* 2014). TGFβs ligand- receptors stimulate phosphorylation of members of R-Smad (receptor activator Smad) including Smad2 and Smad3, to form a complex with Smad4 and transfer to the nucleus where it binds to the promoters and regulates the expression of chondrogenic genes (Ross S. *et al.* 2008).

From studies determining the intracellular signalling chondrogenic pathways, it has been deemed that the transcription of Sox9 regulates the chondrogneic differentiation processes (Kawakami Y. *et al.* 2006). Following the condensation of differentiated and matured chondrogenic-differentiated MSCs, the regulatory effect of Sox9 is subjected to

other subgroups of Sox proteins, i.e.Sox5 and Sox6. (Ikeda T. *et al.* 2005; Hidaka C. *et al.* 2008).

The importance of Sox9 during chondrogenesis was reported in different studies. Its physiological role has been demonstrated in specific genetic disease such as campomelic dysplasia. In this disease, the heterozygous mutations in the Sox9 gene caused an incomplete development of the skeleton (Ikeda T. *et al.* 2005). In addition, retroviral transfection of Sox9 on dedifferentiated chondrocytes increased the production of chondrogenic matrix such as Collagen type II and GAG, thus demonstrating the importance of the Sox9 gene (Hardingham T.E. *et al.* 2006).

It needs to be highlighted here that extra cellular matrix (ECM) components play an important regulatory role during chondrogenesis. Certain proteins such as endoglin and betaglycan (βglycan) that bind to growth factors such as TGFβs and present them to their associated receptors on the cell surface enhances the chondrogensis process (DeLise A.M. *et al.* 2000). ECM has dynamic and functional roles in transmitting signals from surrounding environment to the cell cytoskeleton (outside-in) or vice versa to disseminate signals (inside-out) through integrins (Harburger D.S. *et al.* 2009). MSCs in condensation phase produce ECM that mainly contain collagen type I, fibronectin, and tenascin. During chondrogenic differentiation, the major proteins produced by ECM are collagen type II and proteoglycans such as aggrecan (DeLise A.M. *et al.* 2000).

During limb bud chondrogenesis in embryo, MSCs undergo different cell fates; they either differentiate to articular cartilage or to the shaft of the limb. The maturation process in the first group of cell undergoes abrupt arrest, and produce cartilaginous ECM contains collagen type II, IX, XI, VI, and aggrecan. It is only in pathological situations such as OA, that articular chondrocytes express hypertrophic factors as the

result of the disruption in the maturation process (Fig. 1-5). MSCs that are differentiated into cartilage producing cells, that produces the rest of the limb including the growth plate, resume proliferation and continues to produce matrix components including Col X, alkaline phosphatase, and express receptors for Ihh, PHrP, BMP-6, matrix metalloproteinase 1, 3, 9, and 13. Terminally matured chondrocytes then finally undergo apoptosis (Drissi H. *et al.* 2005; Hidaka C. *et al.* 2008). TGFβ, PTHrP Ihh/Shh inhibits hypertrophic maturation of chondrocytes through their downstream signalling pathways (Derynck R. *et al.* 2008).

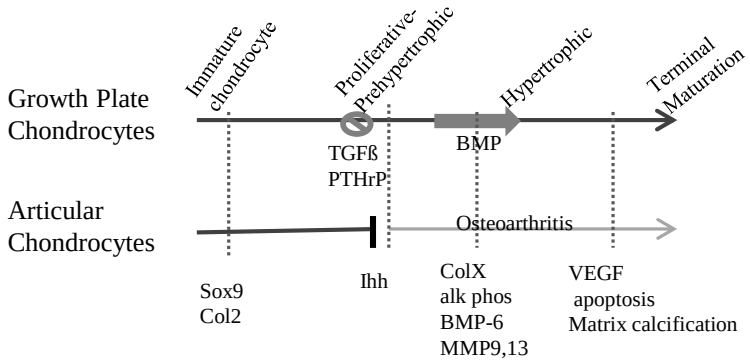


Figure 1-6 Permanent cartilage vs. hypertrophic cartilage pathways

During limb formation MSCs undergo different fate during chondrogenesis i.e.: Growth plate chondrocyte or articular chondrocyte (Drissi H. *et al*. 2005).

1.5 Objectives of this thesis

General objective: To investigate the use of alginate in chondrogenic differentiation of mesenchymal stromal cells and its usefulness in cartilage repair.

Specific objectives:

- To compare the chondrogenic differentiation potential of MSCs in alginate beads, pellet, and monolayer cultures using chondrogenic medium.
- To characterize chondrogenic differentiated MSCs (CMSC) by identifying the morphological, biochemical, gene analysis, cell viability/proliferation changes.
- To study the gene expression levels of adhesion molecules during the chondrogenic differentiation process of the human MSCs.
- To study the repair outcomes of transplanting chondrogenic and undifferentiated MSC on focal chondral defects of rabbit knees.
- To determine the efficacy of alginate without cells in the repair of damaged cartilage, using rabbit knee model.

1.6 Hypotheses

Hypothesis 1: MSCs embedded in alginate beads supported by chondrogenic medium will produce and sustain chondrogenic phenotypic expression similar to cells derived from articular cartilage.

Hypothesis 2: Cell adhesion molecules of NCAM and N-cadherin will not be expressed in a differentiated chondrogenic alginate model.

Hypothesis 3: The alginate constructs with condrogenic differentiated MSCs augments the repair outcomes when applied to damaged articular cartilage.

Hypothesis 4: Alginate may be sufficient in promoting cartilage tissue repair without the use of MSCs.

2 CHAPTER 2: Method development

2.1 Bone marrow isolation

2.1.1 Rabbit

Animals (3-5 months old, 2.5–3.3 kg) were sacrificed in accordance with the guidelines of the Animal Care and Use Committee and, institutional review board of University of Malaya (Reference number: OS/10/11/2008/0611/HD (R)) using a high dose of Nembutal intravenously. The long bones of the animal (Tibia, Femora, and Humorous) were collected under sterile conditions. The samples were kept on iced cooled 1XPBS (PH 7.2) supplemented with 4% antibiotic/antimycotics (Invitrogen). In a laminar flow hood, the bones were crushed using a bone cutter leaving the bone marrow to be collected, diluted, and mixed with the same volume of 1XPBS supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) by pipetting. Four ml of the diluted bone marrow was layered on 3ml of Ficoll Paque (Amersham Biosciences, Uppsala, Sweden) in a 15ml centrifuge tube and centrifuged in 2200 rpm for 25 minutes.

The mononuclear layer (Fig. 2-1A) was collected using a pipette, washed in 1XPBS containing 1% Penicillin/Streptomycin and centrifuged at 1100 rpm for 5 minutes (Fig. 2-1B). The pellet was then re-suspended in the growth medium (DMEM-LG supplemented with 10% FBS and 1% Penicillin/Streptomycin) and cultured in 75ml culture flasks (Nunc, Rockside, Denmark) in a humidified incubator (37 ^{o}C, 5% CO_2).

2.1.2 Human

Human bone marrow samples were obtained from adult healthy individuals (male, age=21$\pm$2.6 years) whom were undergoing fracture fixation involving the long bones. Approval to collect human samples was with ethics approval from University of Malaya

Medical Centre Ethics Committee (reference No. 472.95). Bone marrow samples were collected in a sterile 3ml BD Vacutainer blood tubes (K2 EDTA, BD franklin Lakes NJ USA) by dedicated orthopaedic surgeons and was kept at 4^{o}C until isolation was performed.

The bone marrow samples were diluted with an equal volume of 1XPBS containing 1% antibiotic/antimyotics and mixed thoroughly. The mononuclear layer was isolated with the same method as described above.

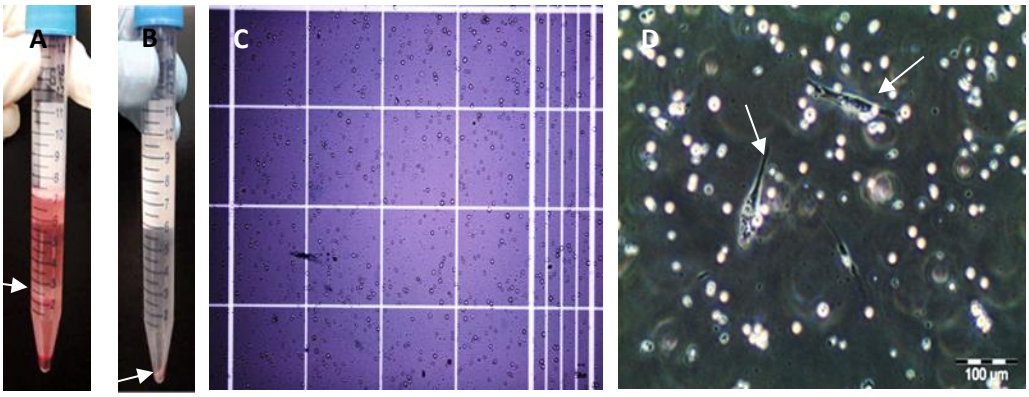


Figure 2-1 Bone marrow mononuclear cells

A. Mononuclear layer (arrow).
B. Mononuclear cells after washing with PBS.
C. Mononuclear cells under light microscopy 10X.
D. (Day 3) P0 fibroblastic shape HMSCs attached to the culture flask (arrow), phase contrast microscopy10X.

2.1.3 Cell culture and expansion

The isolated mononuclear cells were cultured in a 75ml culture flask and supplemented with growth medium (DMEM-LG supplemented with 10% FBS and 1% penicillin/streptomycin) which was replaced every three days. With the first change in medium, the non-adherent cells were washed out. The adherent cells (Fig. 2-1D) were expanded and passaged until passage 3 (P3) before being characterized**.**

2.1.4 Passaging MSCs

Cells were cultured until they reached 70-80 % confluency (Fig. 2-3B). Before adding 3ml of TrypLE^{TM} (Invitrogen), the remaining FBS was removed from the culture by discarding the medium and washing with 1XPBS containing 1% antibiotic/antimycotic, so that it would not inhibit the enzyme during tripsinization. After 3 minutes incubation with TrypLE at 37 ^{o}C, the cells were monitored under a phase contrast microscope to verify detachment of the cells (about 90%), before adding 4-6ml DMEM containing 10% FBS to the culture to neutralize the active enzyme in TrypLE. The remaining cells were then detached completely from the surface using a cell scrapper and centrifuged in 15ml propylene tubes at 1100 rpm for 5 minutes. The pellet was re-suspended in 1ml of medium. Cell count was conducted using a haemocytometer and the cells were sub cultured in 75ml culture flasks with a density of 3000-5000 cells per cm^2. The medium was changed every 3 days.

2.1.5 Cell counting and viability assessment

The viability of cells was determined by TrypanBlue exclusion dye method. While live cells do not absorb the dye (Fig. 2-2A), it penetrates the cell membrane of dead cells staining them blue (Fig. 2-2B and C). 10 µl of the cell suspension was mixed with 10µl of Trypan Blue (Sigma) and mixed by pipetting. 10µl of the mixture of the cell/dye was placed in each chamber of a haemocytometer which had already been cleaned with ethanol and wiped with a lent-free tissue. The cells were counted in 5 squares of each chambers (the corners and the central squares, total 10 squares from up and down chambers) (Fig. 2-2) and calculated using the formula: $N= X/S*10^4*D$ in which, N= Total number of cells, X= Number of counted cells, S= Number of the squares, D= Dilution factor. To avoid overlapping of the cell count in the small squares, cells were only counted on the left and bottom borders (Fig. 2-2).

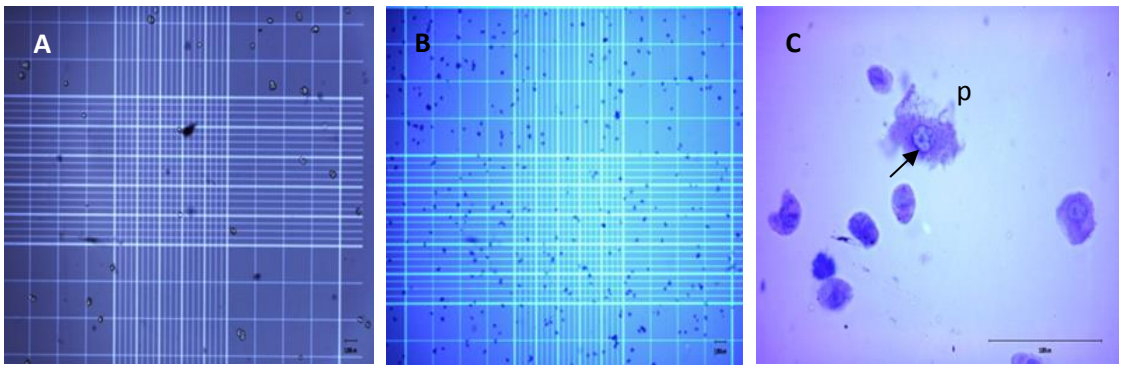


Figure 2-2 Cell viability TrypanBlue exclusion

A. Live cells do not pick up the TrypanBlue stain, and look glistening under microscope 4X.
B. Dead cells stained blue with trypanBlue staining 4X.
C. Higher magnification of dead cells stained with TrypanBlue, 40X arrow= Nucleus, p=Phyllopodia.

2.1.6 Cryopreservation

In order to preserve the cells for long time, the cells were tripsinized in sub-confluency and washed with 1XPBS containing 1% Penicillin/Streptomycin (Gibco) by centrifugation 1100 rpm for 5 minutes. A mixture of 10% DMSO (Sigma) in FBS (Gibco) was prepared and added to the cells after discarding the supernatant thoroughly. The cells were mixed with the cryo-medium by gentle pipetting and transferred to 1ml cryo-tubes (TPP-Germany), within a cryo freezing container (Nalgene®, Mr Frosty) and were placed at -80^{o}C overnight before being transferred into liquid nitrogen tanks (- 196 ^{o}C) (Freshney R.I., 2005).

2.2 MSC Characterization

The plastic adherent mesenchymal stromal cells (MSCs) (Horwitz E.M. *et al.* 2005) were further characterized based on the minimal criteria to define MSCs by the International Society for Cellular Therapy (Dominici M. *et al*. 2006). General morphology was studied using phase contrast microscopy (Fig. 2-3).

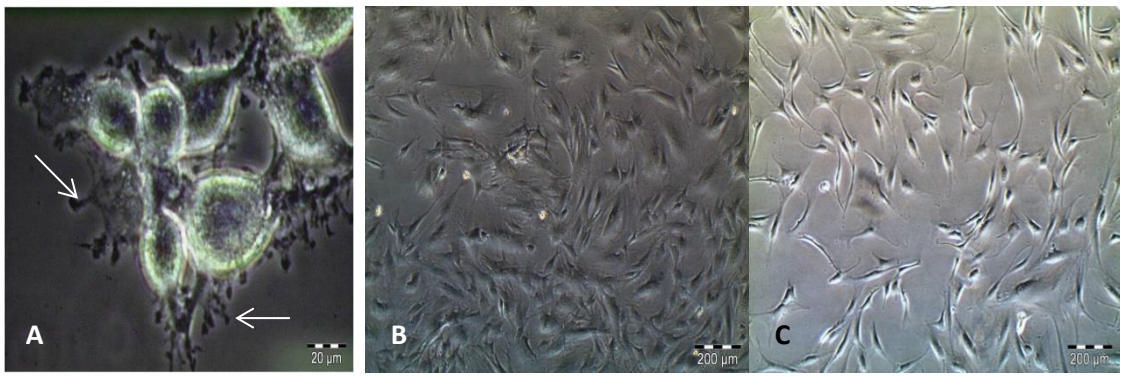


Figure 2-3 Plastic adherent and fibroblastic shape of MSCs, phase contrast microscopy

A. Cell protrusions (arrow) adhere on plastic surface as early as 2 hours after seeding. Rabbit MSC, P3 40X.
B. Rabbit MSCs at P1, 70-80 % confluency 4X.
C. Human MSCs at P3 4X.

The presence or absence of CD markers: CD29,CD44, and CD45 were studied in both human and rabbit MSCs using immunocytochemistry , and a panel of antibodies for CD markers: CD105,CD90,CD73,CD44,CD45, and CD34 were studied in human MSCs using flow cytometry:

2.2.1 Immunocytochemistry

Expression of CD markers in human and rabbit stem cells were studied using immunocytochemistry for CD29 anti-Human [P4G11] Abcam, mouse mAb to integrin beta1 (Abcam, Cambrige, UK) dilution 1/50, CD44 (Rat mAb (A020, CALBIOCHEM©, Merck, Germany) anti rabbit dilution 1/400, and CD45 (mAb, mouse anti Rabbit, AbD Serotec, Oxford) dilution of 1/100 as a negative marker (Fig. 2-4).

MSCs in P3 were cultured on chamber slides, at cell densities of 3000-5000 cells per cm^2. In subconfluency or when confluency is achieved, the cells were washed with phosphate buffer saline (PBS), fixed in 10% formalin for 20 minutes, and stained according to the manufacturer's instructions provided in the DAKO kit: Cells were

treated for 5 minutes with peroxidase block (0.03% hydrogen peroxide containing sodium azide) and rinsed with Tris-Buffered Saline (TBS) pH 7.6 or distilled water. Primary antibodies were applied to the test wells for 30 minutes. In the control wells, the cells were covered with TBS as a negative control for 30 minutes. The cells were then rinsed with TBS before peroxidase conjugated secondary antibody (peroxidase labelled polymer: goat anti-mouse immunoglobulins) was applied to the test as well as the control wells for another 30 minutes. After washing with TBS, the cells were stained with the Substrate-Chromogen (containing hydrogen peroxide and a preservative) plus DAB solution (3, 3' diaminobenzidine chromogen solution) for 5-10 minites. Cells were then washed by distilled water and counter stained with haematoxylin for 2 minutes and the nucleus staining was enhanced in bluing agent (10 dips). The slides were then dehydrated in an ascending series of ethanol, cleared in xylene, and mounted with DPX.

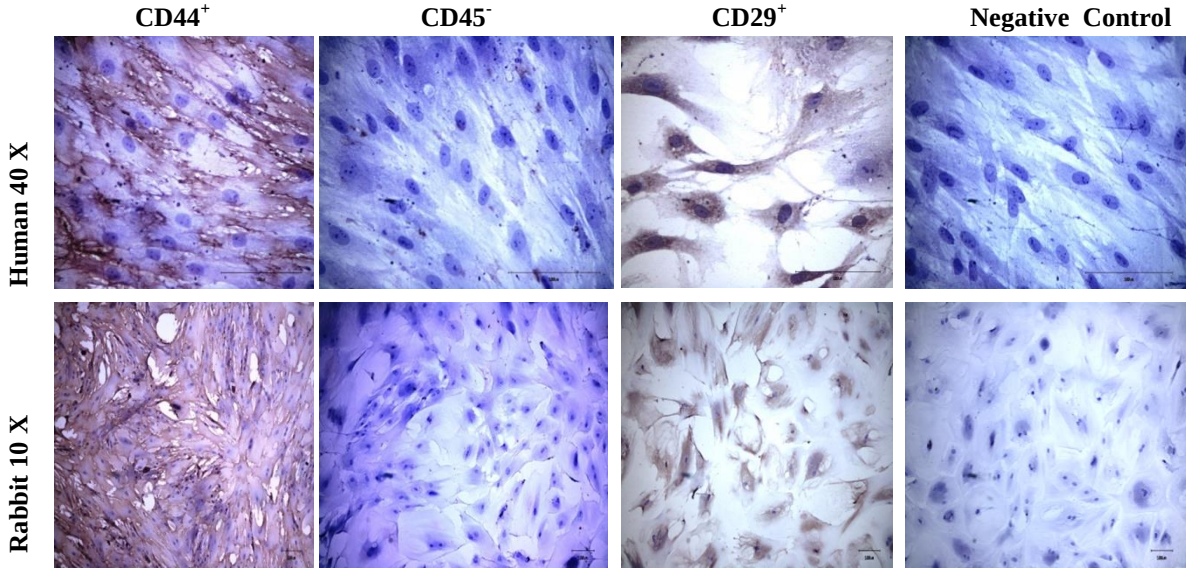


Figure 2-4 Immunocytochemistry for CD markers

MSCs in human and rabbit showed positive for CD44 and CD29 and negative for CD45. Upper panel human MSCs, Lower panel rabbit MSCs.

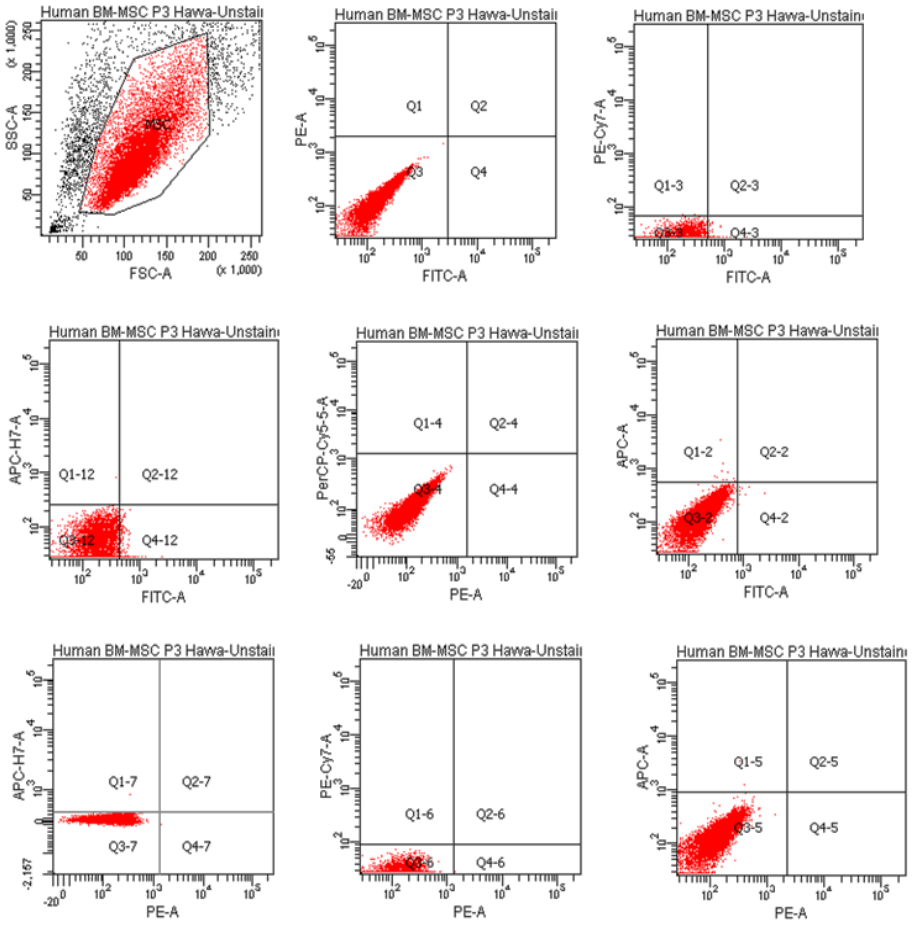
2.2.2 Flow Cytometry

Expression or the lack of expression of a panel of antibodies was studied in human MSCs using flowcytometry analysis: CD105 (Endoglin), CD73, CD45 (leukocyte common antigen), CD44 (Hyaluronic acid receptor), CD34 (haematopoietic progenitor cell marker), and CD90. Each antibody (Mouse anti-Human) was conjugated with a special fluorochrom: Percp-Cy 5.5 CD105, APC CD44, FITC CD73, PE-CyTM7 CD90, PE CD34, and APC-H7 CD45.

BD^{TM} CompBead plus (7.5um) particle set was used as a negative control for the sample. The beads were prepared according to the manufacturer protocol: The BDTM CompBead plus was vortexed before use. For each fluorochrom conjugated antibody use in the experiment a 12x75 mm sample tube (Falcon) was labelled. 100ul of PBS as staining buffer was added to each tube. One full drop (60ul) of each BD CompBead was added to each tube and vortexed. The samples were incubated in dark chamber at room temperature for 15-30 minutes. Using BD^{TM} CompBead plus Negative Control (BSA) beads, the voltage of the flow cytometer instrument was set for MSCs experiment. 2ml PBS was then added to each tube and pellet by centrifugation at 200xg for 10 minutes. The supernatant was aspirated and discarded thoroughly using a fine-tip pipette. Another 0.5ml of PBS was added to each tube and mixed using a vortex. Each tube was run separately on the flow cytometer. Each single bead population gated on FSC (forward-light scatter) and SSC (side-light scatter) characteristics.

Sample preparation for flow cytometry: MSCs were tripsinized at P3. The Cells ($500x10^3$) were suspended in 100µl PBS. Each mAbs antibody stock was diluted to an optimum concentration for 10^6 cells and the antibodies were added accordingly: CD73 FITC 5µl, CD34 PE 10µl, CD105 PCP 5.5µl, CD44 APC 10µl, CD 45 APC 5µl,

and CD90 PECY 5µl. the samples were kept for 15 minutes in dark, incubated on ice before 2ml PBS was added and centrifuged 200g for 10 minutes. The supernatant was discarded and 500µl of PBS was added and vortexed. Samples were kept in dark and on ice before flow cytometry analysis. Gating was performed using forward and side scatter to exclude cellular aggregates and debris. At least 10000 events per sample were analysed by the BD FACSDiva software (BD Biosciences).



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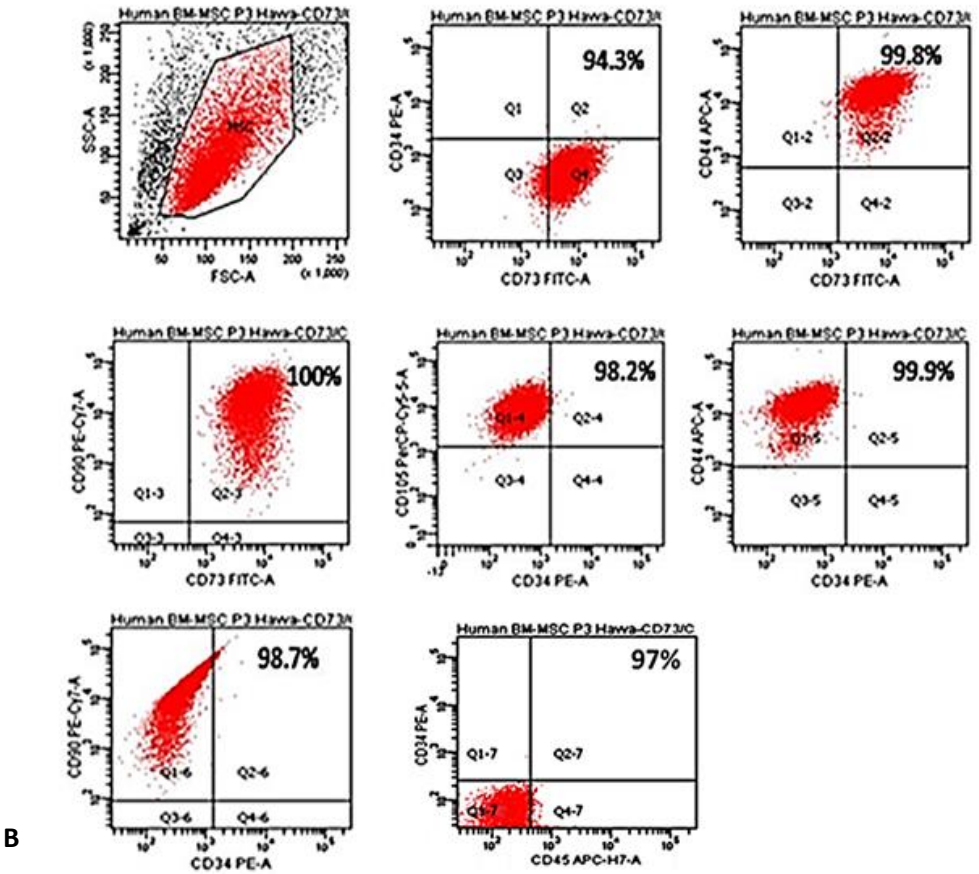


Figure 2-5 Co-expression of CD markers in HMSCs using flow cytometry

A. Un- stained sample negative control dot-plots.
B. HMSCs were stained with antibodies against the indicated antigens labelled on each axis. Representative dot plots were derived from gated events and displayed as combination of forward and side light scatters (FSC and SSC) on a log scale. FSC correlates with particle size while SSC indicates granularity or internal morphology of cells or particles. Cells/events in Q2 are positive and in Q3 are negative for both axes. Q1 is positive for Y and negative for X, Q4 is positive for X and negative for Y (Appendix I).

Table 2-1 Percentage co-expression of CD markers in HMSCs

Antigens	CD markers (%)
CD73+ and CD34-	94.3
CD73+and CD44+	99.8
CD73+ and CD90+	100
CD105+and CD34-	98.2
CD44+and CD34-	99.9
CD90+and CD34-	98.7
CD34- and CD 45-	97

2.2.3 Tri-lineage differentiation

Multi lineage differentiation of mesenchymal stem cells (MSCs) were investigated in rabbit and human stem cells, by inducing osteogenic, adipogenic and chondrogenic lineages as follow:

2.2.3.1 Osteogenesis

MSCs were cultured in chamber slides (3000-5000 cells/cm^2). Upon confluency, the culture was supplemented with osteogenic medium (Gibco) up to 21 days. The medium was changed every three days, and cell morphology was assessed using phase contrast microscopy at three-day intervals. To verify the calcium deposition after 21 days, the cells were stained with Alizarin Red S (Sigma). To demonstrate this, the medium was removed and the cells were washed with 1XPBS, and fixed with formalin 10% for 20 minutes. The fixative was removed and the cells were stained with 2% AlizarinRed solution (Appendix B-e) for 10 minutes. The dye was then removed from the culture and the cells were washed with 1XPBS once or twice. Images were captured at this stage using a phase contrast microscopy. Alternatively after proceeding with dehydration in ascending graded series of ethanol, clearing with Xylene and mounting with DPX (Fig. 2-8) images were captured through a light microscope.

2.2.3.2 Adipogenesis

MSCs were cultured in chamber slides and the cells were supplemented with growth medium until reaching confluency. The cells were then supplemented with defined medium (Gibco adipogenic medium) until day 21. The medium was change every 3 days. On day 21 the cells were washed with PBS and fixed with 10% formalin for 10-20 minutes followed by rinsing with distilled water twice. The cells were then

incubated in 60% isopropanol for 5 minutes at room temperature and stained with Oil Red working solution (Appendix B-f) for 10 minutes. The dye was removed and the cells were washed four times with ddH_2O. Images were taken at this stage through light microscopy while the cells were covered with distilled water (Fig. 2-8).

2.2.3.3 Chondrogenesis

In order to test the quality of defined medium for chondrogenic differentiation of MSCs it compared with standard medium (STEMPRO® Chondrogenesis Differentiation Kit, Gibco-Invitrogen) with two types of cell aggregates micromass and pellet cultures, in two groups of induced (chondrogenic medium) and non-induced (growth medium). Production of cartilaginous extracellular matrix or proteoglycans verified using Safranin O Fast green and Alcian Blue staining (Appendix B).

Chondrogenic medium (defined medium): Chondrogenic medium was prepared according to Gregory C. A. & Prockop D. J. (2007). It included DMEM high glucose (4.5mg/ml D-Glucose) with sodium pyruvate (110µg/ml) (invitrogen), ITS-A 50 mg/ml (1X) (invitrogen), L Ascorbate 2 phosphate (50µg/ml) Sigma, TGFβ3 10ng/ml (Invitrogen), Dexamethason 100nM ($1x10^{-7}$M; Sigma), Penicillin/Streptomycin 100µg/ml (Invitrogen), L Proline 40µg/ml (Sigma).

Micromass culture: MSCs at P3 were seeded in 5µl drops in a cell concentration of 2 million cell per ml in a 24 well culture dish (2 micromasses per well), and incubated in 37^oC and 5% Co_2 for 2 hours, before adding media. The wells divided in different groups of control or test. The control group was cultured using growth medium, while

the test groups were supplemented either by commercial chondrogenic medium (Gibco-Invitrogen) or by defined medium (6 well per group). The media were changed every 3 days. After 14 days the micromasses stained with Safranin O and Alcian Blue (Fig. 2-6).

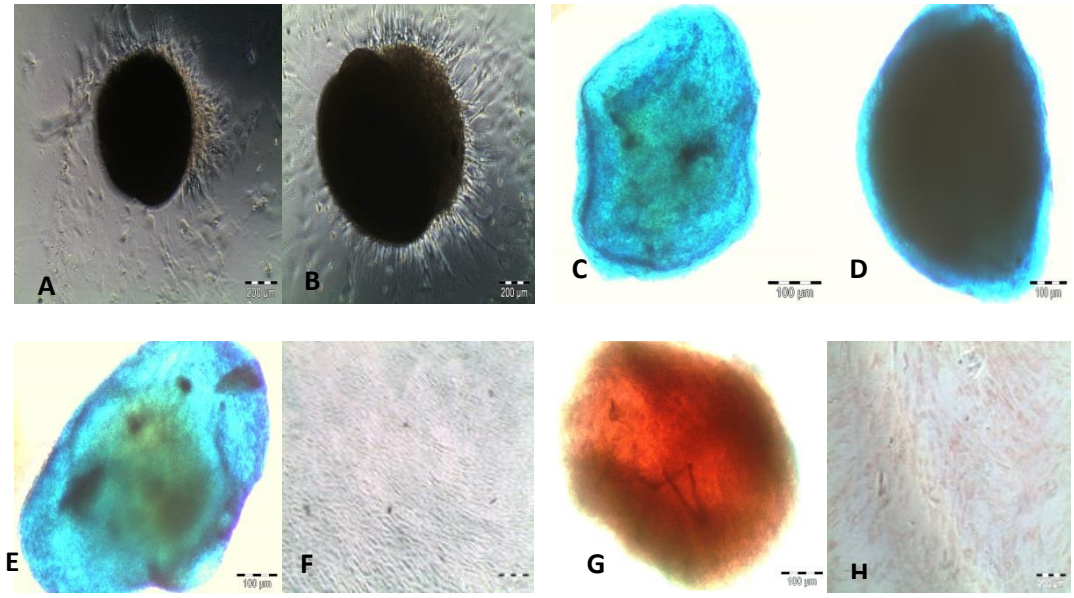


Figure 2-6 Chondrogenic differentiation of human MSCs in Micromass culture

A. Day 6 cultured in standard medium, phase contrast microscopy 4X.
B. Day 6 cultured in defined medium, phase contrast microscopy 4X.
C. Day 14 cultured in standard medium stained with Alcian Blue10X.
D. Day 14 cultured in defined chondrogenic medium stained with Alcian Blue 10X.
E. Day 14 cultured in defined medium, Alcian Blue staining 10X.
F. Day 14 negative control cultured in growth medium stained with Alcian Blue 4X.
G. Day 14 cultured in defined chondrogenic medium Safranin O staining 10X.
H. Day 14 negative control cultured in growth medium stained with Safranin O 4X.

Pellet culture: The chondrogenic medium was then tested with pellet culture of human and rabbit MSCs for chondrogenesis. $250x10^3$cell harvested at p3 and centrifuged at 1100 rpm for 5 minutes, the supernatant discarded and the pellet supplemented with 2ml of chondrogenic medium for one month. Medium was changed every 3days. The pellet were then fixed with10% formalin and processed for routine histology. Paraffin sections were stained with Safranin O Fast green (Appendix B). The staining outcome

(Fig. 2-6 & 2-8) compared with positive control samples of human and rabbit cartilages stained with Safranin O Fast green and Alcian Blue (Fig. 2-7).

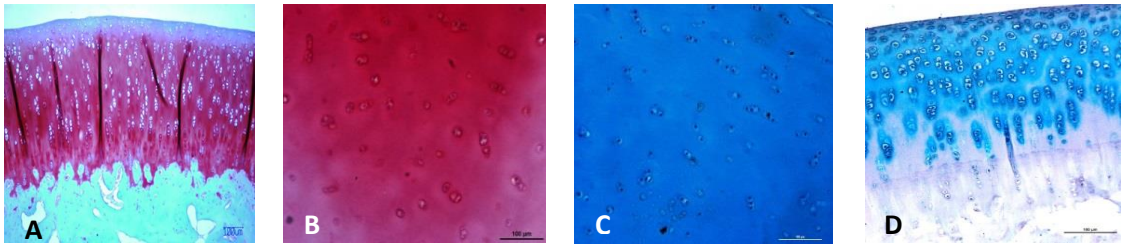


Figure 2-7 Safranin O Fast Green and Alcian Blue staining of human and rabbit Cartilage

A & B. Safranin O Fast Green. Cartilage stains deep red in zones rich in GAG content, bone and other low/no GAG content of cartilage stains green A. Rabbit cartilage 10X, B. Human cartilage 20X.

C & D Alcian Blue staining, cartilage stains blue in zones rich in GAG content. C. Human cartilage 20X, D. Rabbit cartilage 20X

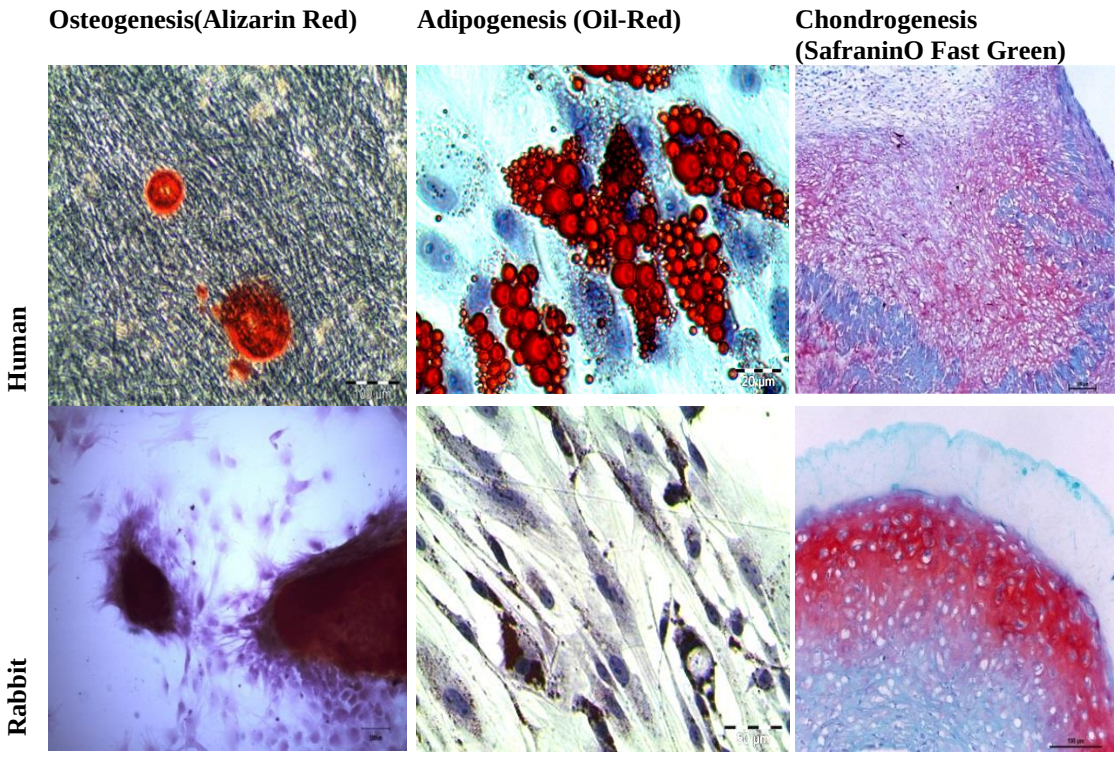


Figure 2-8 Tri-lineage differentiation of human and rabbit MSCs

Mineral deposition in osteogenic cultured stem cells. Human 10X and rabbit 20X. Alizarin Red staining differentiated human MSCs to pre-adipocytes. Oil Red staining, counter stained with Haematoxylin 40X. Rabbit 20X. Chondrogenic differentiation of human 10X, and rabbit MSCs 20X Safranin O Fast Green.

2.3 Proliferation assay

Cell proliferation rate was assessed using AlamarBlue® colorimetric assay (Invitrogen) according to the manufacturer protocol. 10% AlamarBlue were prepared in medium and added to the cells culture, after 3 hours of incubation (37°C, 5% CO_2), 100µl of the medium from each sample and from the 10% AlamarBlue solution as negative control were transferred into a 96 well culture plate (Costar, Corning, NY, USA) (3 replicates). The absorptions of 570nm and 600nm were recorded using Epoch microplate spectrophotometer and Gene 5 data analysis software (Epoch Bio-Tek). The percentage absorption of AlamarBlue was calculated according to the formula from www.Invitrogen.com (Appendix D).

Standard curve: Cells were harvested at P3 and cultured with different cell concentrations at 3 replicates in six well plates for producing a standard curve. On day 3 medium was removed from the cells and replaced by a medium containing 10% AlmarBlue. The absorption of 570 and 600nm were measured by a micro plate reader and a linear line was plotted after data were analyzed by respected formula (Appendix D).

2.4 Gene expression analysis

2.4.1 Primers

Human gene sequences for Collagen type II, aggrecan, Sox9, Collagen type I were obtained from NCBI gene bank. The primers designed using Primer 3 and NCBI software. The selected primers were analysed with the NetPrimer software for secondary structures such as Hairpin, Dimers, Cross-dimers, and Palindromes repeat and run in primer pairs. The chosen primers were checked for speciality with NCBI Nucleotide blast tool: http://blast.ncbi.nlm.nih.gov/

2.4.2 RNA isolation

Cells were tripsinized from monolayer, cell count and viability test with TrypanBlue were performed. The cells then washed with iced cooled PBS and centrifuged at 1100 rpm for 5 minutes and kept in -80°C until further processing. RNA was isolated from cells using SV total RNA isolation system, Promega (USA) according to the manufacturer instructions.

All Pipettors and surfaces were wiped with RNase away spray before use to create a ribonuclease free environment in order to reduce the risk of RNA degradation. Quality and purity of RNA was measured using a NanoDrop 2000 (ThermoScientific, UK) spectrophotometer. The UV absorbance of 280nm for DNA, 260nm for RNA and 230nm for background carryovers (salts, proteins) were measured. The ideal values were placed 1.8-2 for the ratios of 280/260 and 260/230. Lower values show the lower purity of 280/260 and carry over for 260/230 .The integrity of RNA were determined using gel electrophoresis for 28s and 18s ribosomal RNA. The ratio of 2/1 for 28s/18s and the sharpness of the bands (without smears) were considered intact RNA (Fig. 2-9). The RNA was kept in -80°C until further processing to cDNA.

2.4.2.1 RNA quality gel electrophoresis

Before gene expression analysis, integrity of extracted RNA from different experimental groups was evaluated using gel electrophoresis: High integrity and purity RNA was selected to be converted to cDNA, based on gel electrophoresis and spectrophotometer results.

200-500ng RNA was loaded in each gel wells along with loading buffer (Biotium gel Red 6X). The gel was visualized in a transiluminator (Alpha Innotech FluorChem® FC2 Imager).

The MW of human 18S rRNA is 1868 and 28S rRNA 5025. In 1% gel electrophoresis sharp bands of RNA 28S place at about 5kb and 18S at 2kb. With an approximation of 2:1 ratio, the findings indicates that the RNA is intact, the faded and smear bands indicate the degraded RNA (arrow).

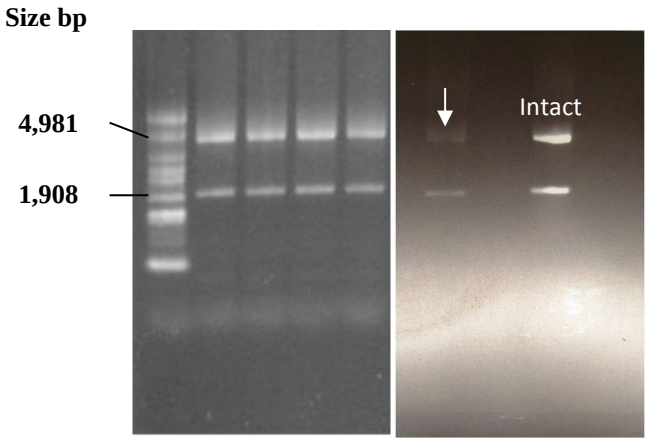


Figure 2-9 RNA quality using gel electrophoresis

A. Gel electrophoresis shows 28S at the level of 4981 bp and 18S bands at the level of 1908 band of ladder. L. RNA marker Promega USA.

B. Comparing a degrading and intact RNA, arrow indicates the fading 28S ribosomal rRNA undergoing degradation.

2.4.2.2 Primer validation and annealing temperature

Normal human chondrocytes (CloneticsTM *(NHAC-kn)* Lonza, Walkersville. Inc. U.S.) embedded in alginate beads for 21 days (to re-differentiate the dedifferentiated chondrocytes after expansion in monolayer) was used as positive control to test chondrogenic markers of Collagen II, Sox9, and aggrecan. RNA for testing housekeeping genes extracted from MSCs in monolayer and for hypertrophic genes of RunX2 and collagen X from MSCs cultured in osteogenic medium on day 12.

2.4.2.3 Gradient PCR

In a gradient series of temperatures, the temperature which was common between primers with the lowest Cq values obtained with RT-PCR or the single and brightest band produced with gel electrophoresis (Fig. 2-10) was chosen as the annealing temperature.

2.4.2.4 Gel electrophoresis for primer assessment

2% agarose in 1XTAE buffer, heated in microwave. The gel was cooled to about 60°C before a 1X of fluorescent dye (Gel Red, Biotium, USA) was added and cast in a mould. A proper comb was inserted to make wells and removed after gel was solidified. The gel was placed in an electrophoresis tank filled with proper amount of 1XTAE buffer (the wells located in the negative side of the tank). cDNA was mixed with 5X loading buffer (Bioline), to make a 1X mixture (4µl cDNA plus 1µl loading buffer) for each well. The first well was loaded with DNA marker. The gel was run at 90 volt for 45 minutes.

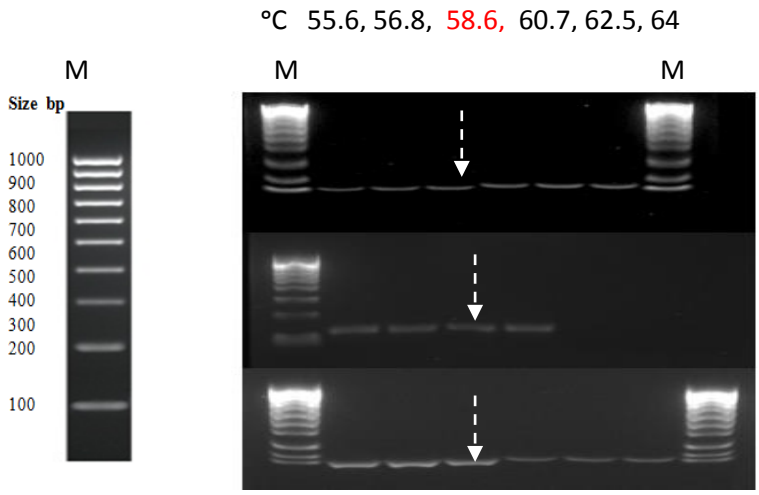


Figure 2-10 Gradient PCR of primers in gel electrophoresis

Gel electrophoresis of 3 primers show the shared annealing temperature (arrows) of 58.6 on gel electrophoresis. M. DNA marker (HyperLadderTM IV, BIOLINE)

2.4.3 cDNA synthesis

In each reaction 100ng RNA were converted to first strand cDNA in 20µl final volume using iScriptTM Reversed Transcription Supermix for RT-qPCR (BioRad) according to the manufactures' protocol.

3 CHAPTER 3

Study 1: Chondrogenic differentiation and characteristics of human bone marrow-derived stromal cells in alginate beads, pellet culture and monolayer

3.1 Study design

In this part of the study, chondrogenic MSCs (CMSC) were characterized after human bone marrow mesenchymal stromal cells were isolated and initially characterized based on "the minimal criteria for defining multipotent mesenchymal stromal cells" (Dominici M. *et al*. 2006) including fibroblastic morphology, cell adherent to plastic surface, tri-lineage differentiation, and expression or lack of expression specific CD markers. The cells were then cultured in 2D monolayer, and 3D (alginate bead, and pellet cultures) in two groups: chondrogenic differentiated MSCs (CMSC) and undifferentiated MSCs. CMSCs were characterized based on morphological parameters (Histology and electron microscopy), proliferation and viability tests and biochemical analysis (Glycosaminoglycan content or GAG) and compared with the non-chondrogenic (control) group.

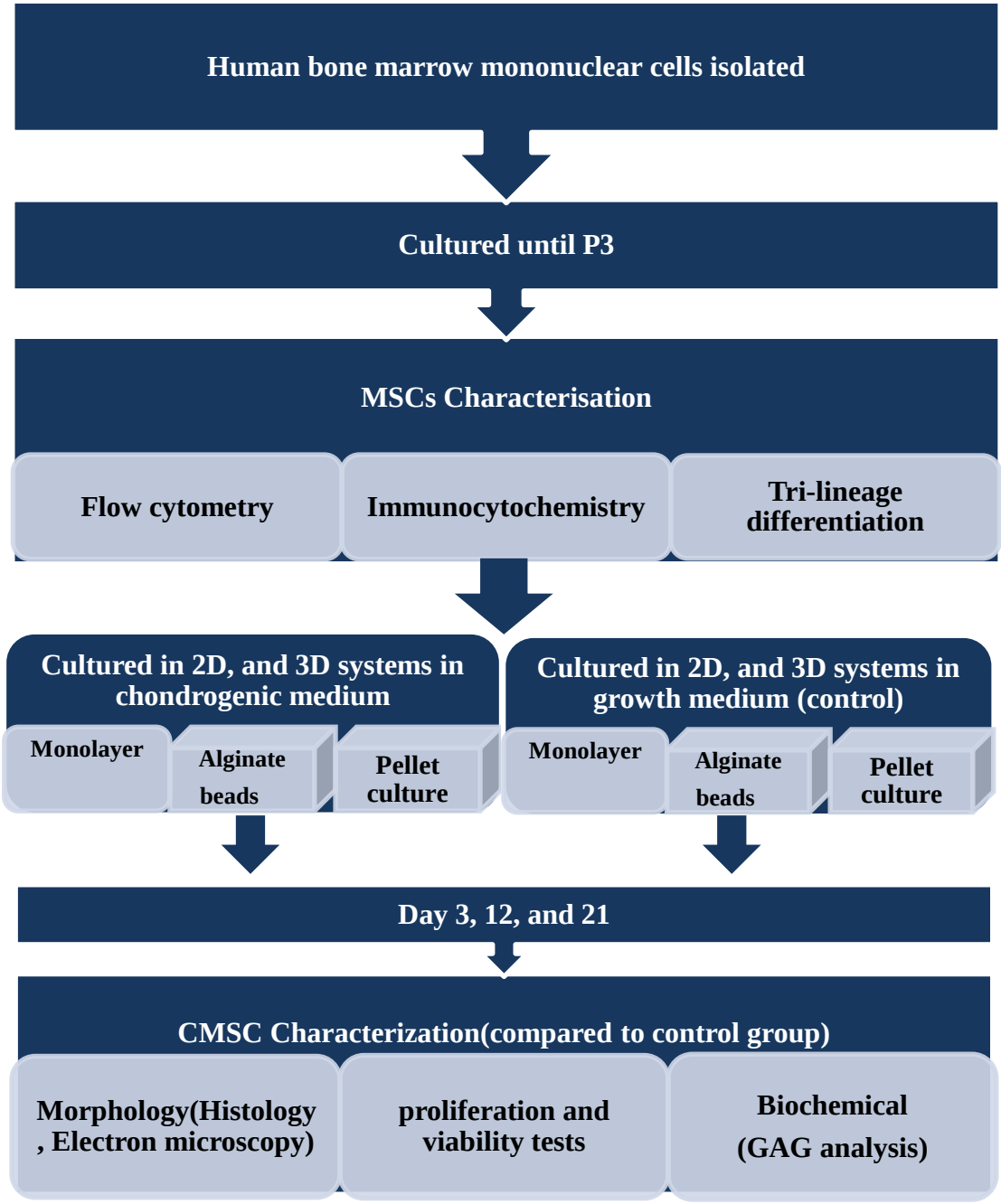


Figure 3-1 Flowchart shows the study design for chapter 3.

3.2 Introduction

Articular cartilage is a unique tissue with a sparse number of chondrocytes responsible for production and maintenance of an extracellular matrix rich in proteoglycans (consisting of a protein core bonded to glycosaminoglycan groups or GAG) and collagen fibrils (mainly type II) (Kheir E. *et al*. 2009). For characterization of an engineered construct it is mandatory to study the main component of articular cartilage such as collagen type II and GAG. GAG are carbohydrate polymers that form building blocks of large aggregating proteoglycans, (Lodish *et al*. 2004) and play an important role in the extracellular matrix (ECM) of cartilage. GAG provides the mechanical support for articular cartilage (LeRoux M.A. *et al*. 2000), and is commonly used as a marker of functionality for damaged or repaired cartilage. This measure is commonly used in cartilage repair outcome studies (Fan H. *et al*. 2006, Sharma A. *et al*. 2007, Vinardell T. *et al*. 2009, Bekkers J.E.J. *et al*. 2013) or to evaluate the effectiveness of constructs to be applied for cartilage tissue engineering (Enobakhare B.O. *et al*. 1996; Murdoch A.D. *et al*. 2007; Chan C.H. *et al*. 2008; Diekman B.O. *et al*. 2010).

In cartilage, chondrocyte is the only responsible cell type for tissue homeostasis or synthesis and degradation of extra cellular matrix (ECM) (Pearle A.D. *et al*. 2005), therefore a profound understanding of the morphology and physiology of the engineered chondrocyte-like cell is required in order to determine the likelihood of cartilage regeneration outcomes. Although alginate culture system has been shown to provide the ideal microenvironment for evaluating chondrogenesis of mesenchymal stromal cells (MSCs) *in vitro,* the detailed ultrastructural description relating to cellular / matrix interactions of chondrogenic-differentiated MSCs cultivated in alginate has not been studied extensively (Yang I.H. *et al*. 2004; Ichinose S. *et al*. 2005; Xu J. *et al*. 2008; Duggal S. *et al*. 2009; Diekman B.O. *et al*. 2010). Whilst we understand that there are several other issues of alginate and use of MSCs, such as cell-matrix interaction and co-

dimer polymerization issues have not been determined as well, the present study is focused on the ultrastructure aspect of alginate specifically.

In this study following morphological studies using light microscopy and quantitative expression of GAG, ultra structure of non-differentiated and chondrogenic differentiated MSCs was studied using scanning and transmission electron microscopies (SEM and TEM).

3.3 Materials and Methods

Three biological samples of cell or constructs (alginate loaded with cells; N=3) were cultured in chondrogenic medium or growth medium (control group) (DMEM-HG supplemented with 10% FBS and 1% penicillin/streptomycin) in six replicates. Outcome measures used for the end of this part of the study includes testing the viability and proliferation of the cells using AlamarBlue, GAG analysis using Blyscan kit, morphological studies using specific staining of Safranin O Fast Green, and immunohistochemistry for collagen II, collagen I and aggrecan. In addition, Alizarin Red staining was performed to rule out any deposition of mineral at the end of chondrogenesis in 3D cultures. Studies on the cell surface was further conducted using scanning electron microscopy (SEM) in the alginate group following chondrogenic differentiation and was compared to MSCs. Transmission electron microscopy (TEM) was performed on 3D cultures of pellet and alginate beads to study the ultrastructural changes during chondrogenic differentiation.

3.3.1 Pellet culture

MSCs were harvested at P3, with $250X10^3$ cells were centrifuged in a 15ml polypropylene tube at 1100 rpm for 5 minutes. The supernatant was then discarded. The

cell pellets were cultured either with 2ml of chondrogenic or growth medium as control. The pellets were then detached from the tube by flicking the tube, the tubes were then kept in a 5% CO_2, and 37°C humidified incubator with loosed cap in order to permit gas exchange. Medium was changed every 3 days. GAG content of medium was measured by every medium change, i.e.: day 3, day 6, day 9, day12, day 15, day 18 and day 21. Other groups of pellet were cultured for morphological studies using histology. At least 3 samples in each group. On day3, day12, and day21 pellet culture samples were fixed in 10% formalin and processed for histological studies staining with Safranin O Fast Green (Appendix B) or immunohistochemistry (according to DAKO kit instructions).

3.3.2 Cell-alginate constructs

1.2% alginate prepared from low viscosity sodium alginate powder (Sigma-Aldrich) in 0.9% sodium chloride (NaCl) and filtered sterile by a 0.2µm filter. MSCs at P3 were harvested and a concentration of $4X10^6$ per ml (~80000 per bead) in alginate bead (Erickson G.R. *et al*. 2002, Mehlhorn A.T. *et al*. 2006) was obtained before dropping the cell laden alginate into sterile, calcium chloride solution ($CaCl_2$) using a pipette. Alginate bead constructs cross linked in this solution for 10 minutes in 37°C incubator and then were rinsed in 0.9% normal saline 2-3 times, three beads per well transferred to the culture dishes (ultra-low attachment 12 well plates, Corning), and supplemented with 2ml chondrogenic or growth media. The medium changed every 3 days.

3.3.3 Glycosaminogylcan contents

GAG content of the medium in different chondrogenic as well as control groups were analysed on days 3, 6, 9, 12, 15, 18,and 21 (n=6) using Blyscan assay kit (Biocolor, Northern Ireland). The protocol was optimized for this experiment using 500µl of

medium and it was mixed with 1ml of dye on a shaker for 30 minutes at room temperature. The samples then centrifuged at 12000 rpm for 10 minutes the supernatant was discarded and 500µl of dissociation buffer was added to dissolve the pellet using a vortex. The absorption of 656nm was recorded using a micro plate reader (Epoch microplate spectrophotometer). The concentration of each sample was calculated using a plot made of different concentrations of standards (Chondroitin-6-sulphate, shark cartilage). If the GAG content exceeded the highest amount of the standard, then the sample is diluted and the experiment repeated. After subtraction of background GAG, it normalized with the cell number in each sample (GAG/Cell). The data presented as an average of three time points of 3, 12 and 21 days. Data for day 12 and 21 were cumulative data of earlier time points.

Background GAG: The final data were normalized with subtraction of background GAG from the GAG values of experimental groups. In two groups of monolayer and pellet culture, the final GAG calculated after subtraction of GAG from growth medium supplemented with 10% FBS and chondrogenic medium alone respectively. Final concentration of GAG in alginate group was obtained by subtracting the GAG from the incubation of cell-free alginate beads in chondrogenic or growth medium at respective time points similar to the experimental groups.

3.3.4 Cell Proliferation

Cell proliferation was assessed using AlamarBlue colorimetric assay (invitrogen) according to the manufacturer protocol. AlamarBlue (10%) was added to the medium of cells/constructs of both groups of chondrogenic and control on day 0, day 3, day 12, and day 21 and incubated in 37°C and 5% CO_2 incubator. After 3 hours the absorptions of

570nm and 600nm were recorded using Epoch microplate spectrophotometer and Gene5 data analysis software (Epoch Bio-Tek).

3.3.5 Live/Dead viability test

LIVE/DEAD® Viability/Cytotoxicity Assay (Invitrogen –Molecular Probes, Eugene, OR) was performed according to the manufacturer's instructions. The principal of the test is based on intracellular esterase activity of live cells and their plasma membrane integrity, using two fluorescent dyes of Calcein and Ethidium homodimer-1(EthD-1) that produce green and red dyes respectively. Calcein AM primarily is a non-fluorescent dye, once entering live cells it converts to fluorescent Calcein and retained in the cell. While Ethidium homodimer-1 only enter cells with damaged membrane, binds to nucleic acids and produces a bright red fluorescent dye in dead cells.

On day 3 and day 21, cell-alginate beads constructs were washed with PBS and stained with working solutions of 2µM Calein AM and 4 µM EthD-1 at room temperature for 15-30 minutes and viewed with a fluorescent microscope (Nikon Eclipse Ti-U).

3.3.6 Histology and Immunohistochemistry

Samples were fixed with 10% formalin for 1 hour, and processed for routine histology. 5µm of paraffin sections were stained with Safranin O Fast Green, (Appendix B-d), and immunohistochemistry of collagen type II with primary antibodies (Mouse mAb (II-4C11) anti rabbit (CALBIOCHEM©, Merck, Germany), dilution of 1/100. Mouse monoclonal [6-B-4] to human aggrecan Abcam (Abcam Plc, Cambrige, UK), dilution of 1/50 and mouse monoclonal (clone I-8H5) anti-collagen type I (CALBIOCHEM©, Merck, Germany) dilution of 1/100 (Using DAKO kit (DakoCytomation, Glostrup, Denmark) according to the manufactures protocol.

3-Dimentional cultures of chondrogenic alginate and pellet culture were also stained with Alizarin Red S (Sigma-Aldrich) staining (Appendix B-e) to verify any mineral deposition during chondrogenesis.

3.3.7 Ultrastructural studies

Detailed structures of cell surface, extracellular matrix and cytoplasm were studied in undifferentiated MSCs and chondrogenic differentiated CMSCs using scanning and transmission electron microscopy.

3.3.7.1 Scanning electron microscopy (SEM)

Cell surface and matrix deposition in chondrogenic MSCs loaded in alginate bead vs. non chondrogenic MSCs was studied using SEM.

Sample preparation: MSCs were tripsinized at P3 and centrifuged at 1100 rpm for 5 minutes. After discarding the supernatant the resulted pellet of MSCs (day 0) along with CMSC in alginate beads on day 21 rinsed with 1XPBS and fixed with 4% glutaraldehyde in cacodylate buffer for 48 hours 4^{o}C. Samples were then washed in the cacodylate buffer twice for 10 minutes and post fixed in the osmium tetroxide 1% for 1hour at 4^{o}C. The samples were washed in the same buffer three times each for 10 minutes and stored in the buffer overnight at 4^{o}C before washing with distilled water for 3 times. A series of dehydration process was employed: dehydration in ascending series of ethanol, alcohol 30, 50, 70, 80, 90, 95, and 3 changes in 100 each for 10 minutes, followed by a mixture of ethanol-acetone in these ratios: 3:1, 2: 2, 1: 3 each for 15 minute, and then to 3 changes of pure acetone each one for 20 minutes. At this stage, samples were transferred to the CPD machine (Bal-Tec CPD030 (Critical Point Dryer), immersed in the pure acetone.

CPD machine is supplied with CO_2. When the temperature goes down to 9-10°C, the liquid CO_2 flushes the samples and replaces acetone which is drained from the machine. While the acetone is completely replaced by CO_2, the temperature goes up to 31-32°C and CO_2 at this temperature turns into gas and escapes from the samples leaves the samples totally dried.

The samples were then mounted on stubs using double sided tape and coated with gold in a sputter coater machine (Hitachi E-1010 Ion Sputter) for 120 seconds, and viewed with the SEM (Hitachi Scanning Electron Microscope S-3400N).

3.3.7.2 Transmission electron microscopy (TEM)

Ultrastructural details of cells and cell matrix interactions during chondrogenic differentiation of MSCs in alginate beads compared with pellet culture and MSC on day 0 using TEM.

Sample processing: A. Fixation: Samples were fixed with 4% Glutaraldehyde in cacodylate buffer for 24 hours at 4^oC and washed with cacodylate buffer 0.1 M, pH 7.4 (Merck AG, Darmstadt, Germany) 3 times before post-fixation in buffered osmium tetra Oxide 1% (Appendix C) for 2 hours at 4^oC. The samples were washed with cacodylate buffer 2-3times and kept overnight at 4^oC.

B. En-block staining: Samples were washed with ddw water 2-3 times and stained with Uranyl acetate (Agar scientific Ltd. R1043, Essex England) 4% in ddw for 10 minutes and washed with ddw 2-3 times.

C. Dehydration and embedding: Dehydration of samples were performed in ascending series of ethanol 35, 50, each 10 minutes followed by 95 and 100 (3times) each for 15 minutes. Propylenoxid (BDH, VWR International Ltd, Poole, UK) two changes each for

15 minutes. transferred to mixtures of 50% Propylen oxide in Epon (a mixture of Propylen Oxide/Epon 1:1 for) for 1 hour, and 33% (a mixture of Propylen Oxid/Epon 1: 3) for 2 hours. The samples were transferred to the pure Epon (Agar 100 resin kit, Agar scientific Ltd. R1043, Essex England) overnight on a rotary mixer (Ted Pella, Redding, CA, USA) at room temperature.

Fresh Epon was put in the labelled embedding moulds. The samples were adjusted in the right position using a fine forceps. The moulds containing the samples were kept at 37^{o}C for 5 hours and transferred to 60^{o}C overnight to polymerize.

D. Ultra Microtome: In order to cut the samples embedded in resin with ultramicrotomy, glass knives were prepared using glass strips in a Leica knife maker and samples were trimmed manually under a dissection microscope with a razor blade.

E. Semi thin sectioning: 1µm semi thin sections were obtained using a Leica ultramicrotome (Reicher Ultracuts, Leica Microscystem, and Vienna, Austria). The sections were collected in a filtered drop of ddw on a pre- cleaned slide and kept on the hot plate 40^{o}C until they dried. The samples were then stained with filtered Toluidine Blue (Appendix C) for 1-2 minutes on the hot plate 40^{o}C, and then were washed with distilled water and air dried before viewing with light microscopy.

F. Ultrathin sectioning: small plastic boats was glued on the glass knives using melted dental wax or nail polish and filled with double distilled water. The blocks, were sectioned at 70-80nm. The sections were floated in the water and were collected on copper grids (300 meshes) and stained with Uranyl acetate and lead citrate (Agar scientific, UK) (Appendix C). Images were viewed using TEM (Leo Libra 120; Carl Zeiss SMT AG, Oberkochen Germany).

3.3.8 Statistical analysis

Values for GAG analysis and proliferation test demonstrated as mean± standard deviation (SD). The different between experimental groups for proliferation test were studied using non-parametric Kruskal-Wallis H test, and for both GAG and proliferation tests, the different between two experimental groups using Mann-Whitney U test available on the statistical software package SPSS (version 18.0). $P \leq 0.05$ was considered significant.

3.4 Results

3.4.1 Gross morphology

Chondrogenic pellet and alginate beads increased in size by day 21 (Fig. 3-1 A&B). Alginate beads on day 21 looks white in texture, it was firm and easily handled compared to an earlier time point on day 3 with a clear appearance (Fig. 3-1B).

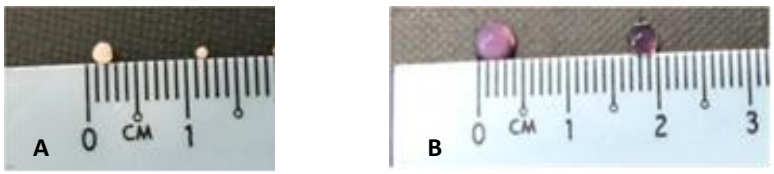


Figure 3-2 Gross morphology of chondrogenic pellet and alginate beads

A. Gross morphology of pellet cultured in chondrogenic medium, left to right: day21, day3.
B. Chondrogenic MSC loaded in alginate bead, left to right: day21, day 3.

3.4.2 Chondrogenic differentiation

Chondrogenic differentiation was verified through histology, immunocytochemisty and biochemical study with GAG analysis. Alizarin Red staining ruled out mineral deposition during chondrogenic differentiation in 3D culture of alginate and pellet on day 21 (Fig. 3-5 B&D), while in monolayer the expression of chondrogenic markers

was very low (Fig. 3-2F, 3-4 C&I) and collagen type I was the prominent protein expressed in chondrogenic monolayer compared to collagen type II (Fig. 3-6, 3-4 C).

3.4.2.1 Histology

Morphological studies of chondrogenic differentiation of MSC in different experimental groups are shown at (Fig. 3-2) Safranin O Fast Green staining of pellet culture on day 21 showed positive GAG stained in red and some green area indicated non-GAG depositions (Fig. 3-2 A). In chondrogenic alginate on day 21 Safranin O Fast green showed mainly red colour an indication of high GAGs production in this group when it compared with control group (Fig. 3-2 B&E).

Safranin O Fast Green staining for GAG detection in monolayer cultured with chondrogenic medium was negative when compared to the control group. Scant staining of cells in the test and control groups mainly indicated the background staining (Fig. 3-2 C&F).

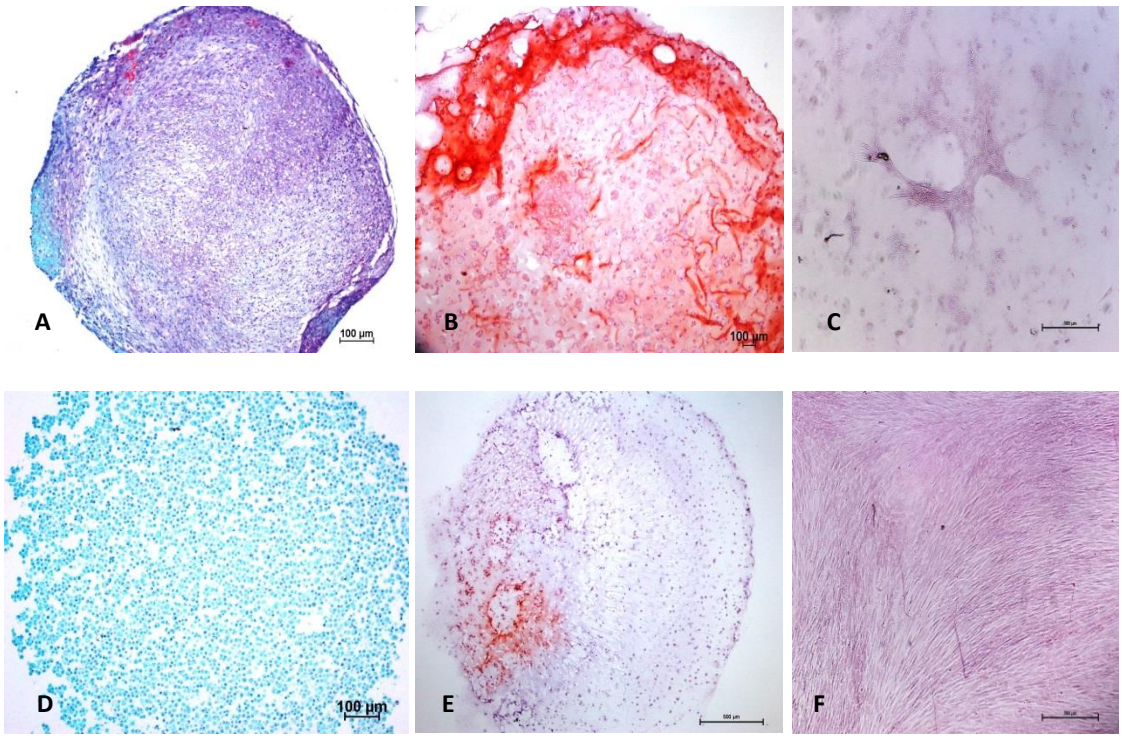


Figure 3-3 Safranin O Fast Green staining of different experimental groups

Upper panel: Chondrogenic groups: A. Pellet culture B. CMSCs in Alginate bead 4X, C. chondrogenic monolayer, day 21, 10X.

Lower panel: Control group: D. pellet culture X10, E. MSCs in alginate bead 4X, F. Monolayer culture, day 21, 10X.

Safranin O Fast Green staining of 3D chondrogenic groups of pellet and alginate culture in different time points was positive from day 12 onward. The intensity of red colour (an indication of abundance of GAG) was increased in alginate group overtime as it was shown in (Fig.3-3), CMSC in alginate group showed similarity with native human cartilage on day 30. The expression of GAG from day 12 onward was more prominent in alginate group compared to pellet culture (Fig. 3-3).

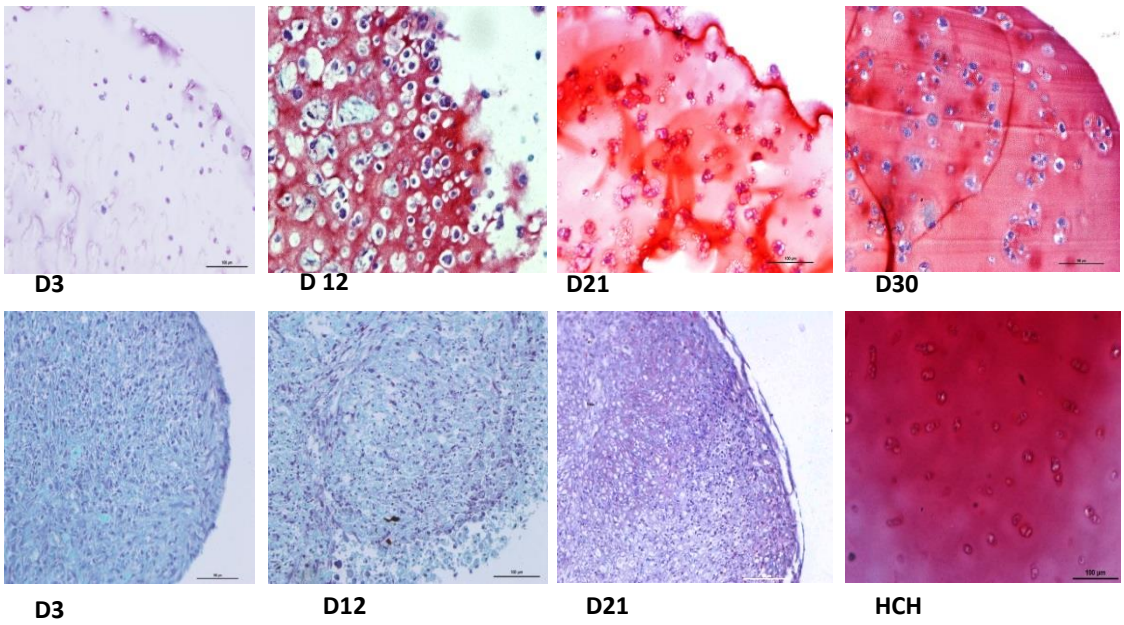


Figure 3-4 Safranin O Fast Green staining of CMSC in alginate and pellet culture over time

Safranin O Fast Green staining of Alginate group (upper panel) and pellet culture (lower panel) on different time points, compared with human articular cartilage (HCH). The intensity of the staining in CMSC alginate D30 was more similar to that of HCH 20X.

The expression of collagen type II in chondrogenic groups of Pellet, monolayer, and alginate showed positive for collagen type II stained with immunohistochemistry method on day 21 (Fig. 3-4 A-C), compared with the negative controls that stained only with secondary antibody (Fig. 3-4 D-F).

Immunohistochemistry study of Aggrecan showed positive staining for chondrogenic pellet and alginate; however the staining had no positive result in immunocytochemistry of chondrogenic monolayer when compared with the negative control (Fig. 3-4 I&L).

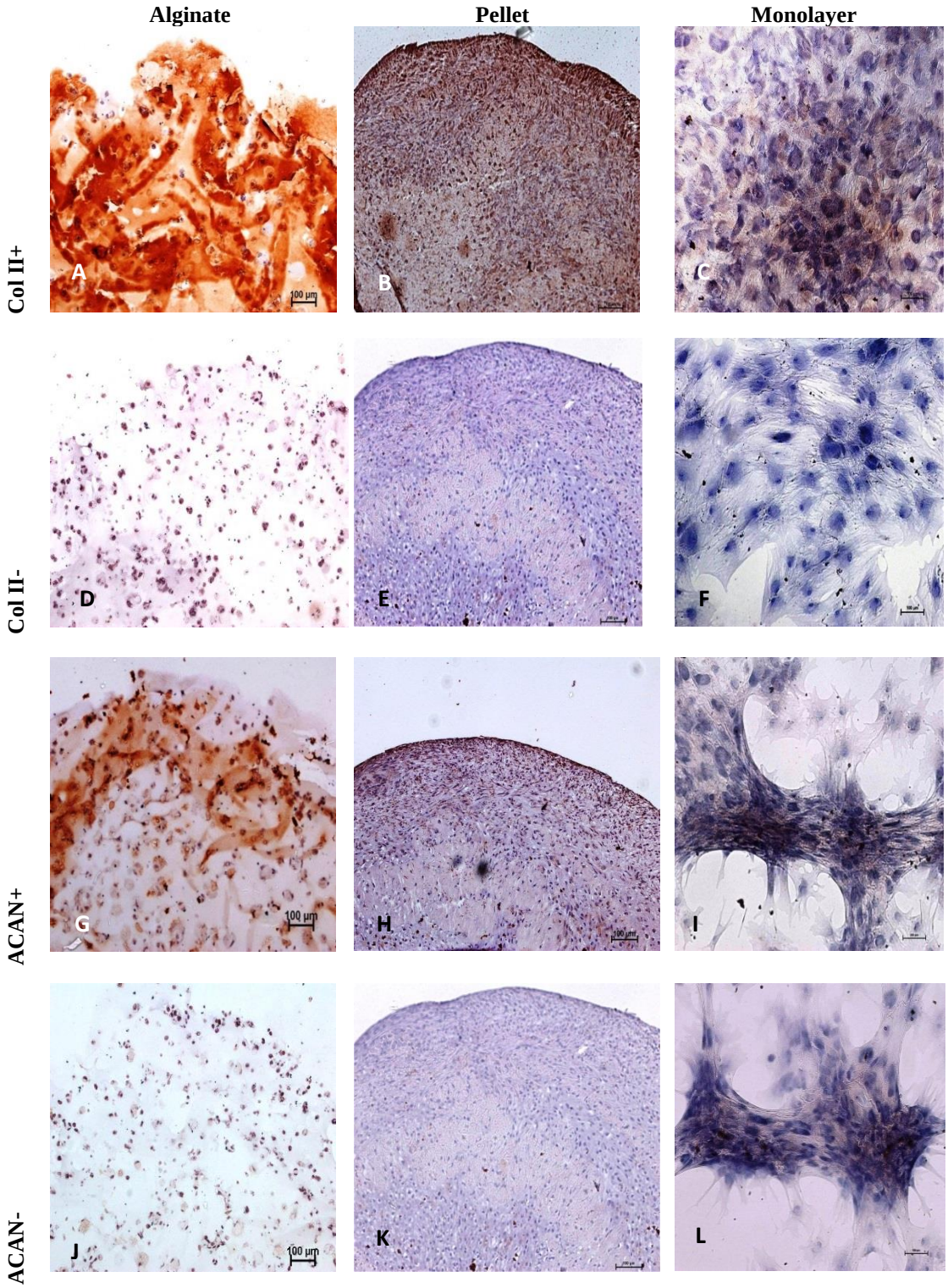


Figure 3-5 Immunochemistry staining of chondrogenic groups on day 21

Collagen type II $^{+}$ (A-C). ACAN^{+} (G-I). Negative control Collagen II^{-} (D-F), ACAN^{-} (J-L) 10X.

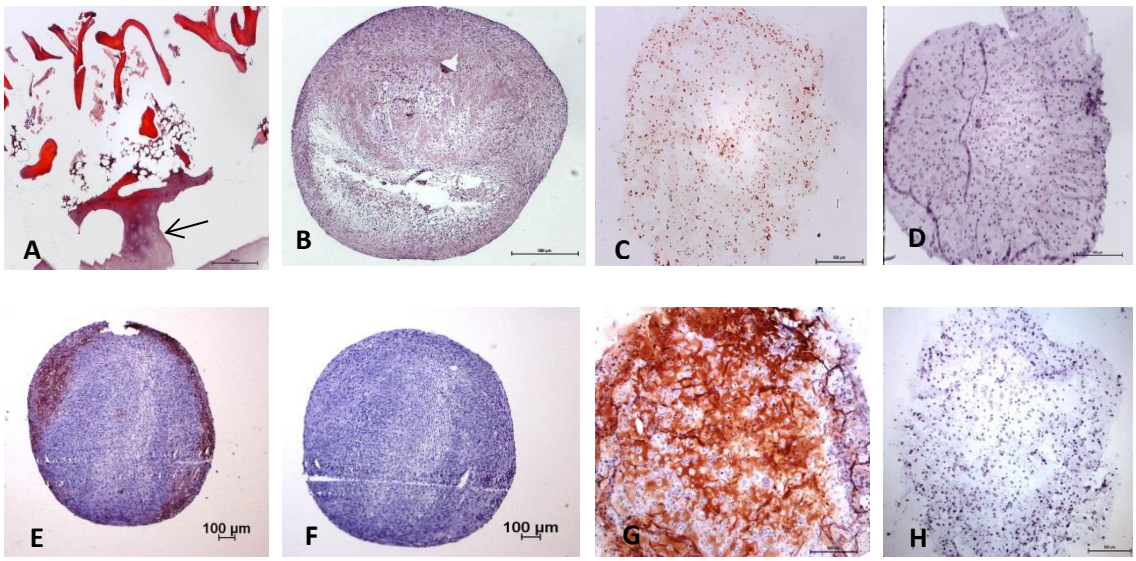


Figure 3-6 Mineral deposition and expression of collagen type I in 3D cultures of pellet and alginate during chondrogenic differentiation

Upper panel: Alizarin Red staining A. Human bone (Red) and cartilage purple (arrow) as positive control 4X, B. chondrogenic pellet day 21, C. CMSC in alginate bead day 21, 4X, D. CMSC in alginate bead day 30, 4X.

Lower panel collagen type I, immunohistochemistry E. Chondrogenic pellet culture day 21 4X F. Pellet negative control, G. CMSC in alginate bead day 21, 4X, H. CMSC in alginate bead negative control 4X.

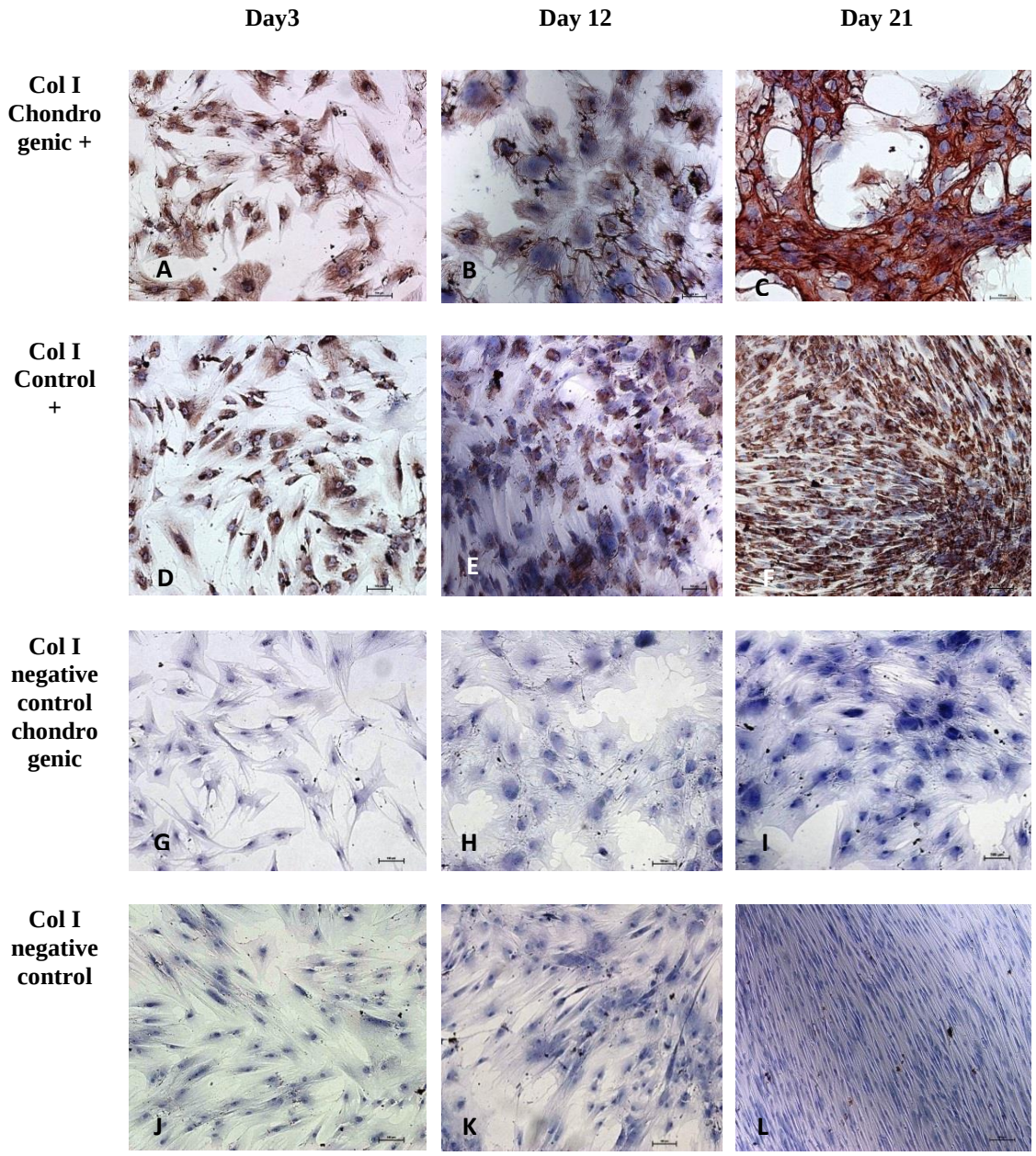


Figure 3-7 Expression of collagen type I in 2D monolayer during chondrogenic differentiation using immunocytochemistry

Premature hypertrophy and increase in collagen type I in chondrogenic group can be seen on day 12 and day 21 (B &C) compared with control group (E & F). G-I negative control for chondrogneic group. J-L negative control for control group, 10X. (+) indicates staining with primary and secondary antibodies, negative control samples stained only with secondary antibody.

3.4.2.2 GAG analysis

Biochemical test for GAG content of supernatant in different experimental groups showed no GAG in the medium of monolayer cultured in chondrogenic medium as well as all control groups of alginate bead, pellet culture and monolayer.

In the chondrogenic groups, there was no significant difference between GAG content of chondrogenic alginate beads and pellet culture on day 3 as it was shown by Mann-Whitney test ($p>0.05$). However at the other time points, day 12 and day 21 alginate beads showed significantly higher amount of cumulative GAG than pellet culture (Fig. 3-7; $p<0.01$).

In the alginate group, GAG content (GAG per cell number) significantly increased overtime from day 3 to day 12 ($p=0.01$), but in pellet culture there was no significant different between day 3 and day 12 GAG ($p>0.05$). GAG content in both group significantly increased from day 12 to day 21 (Fig. 3-7).

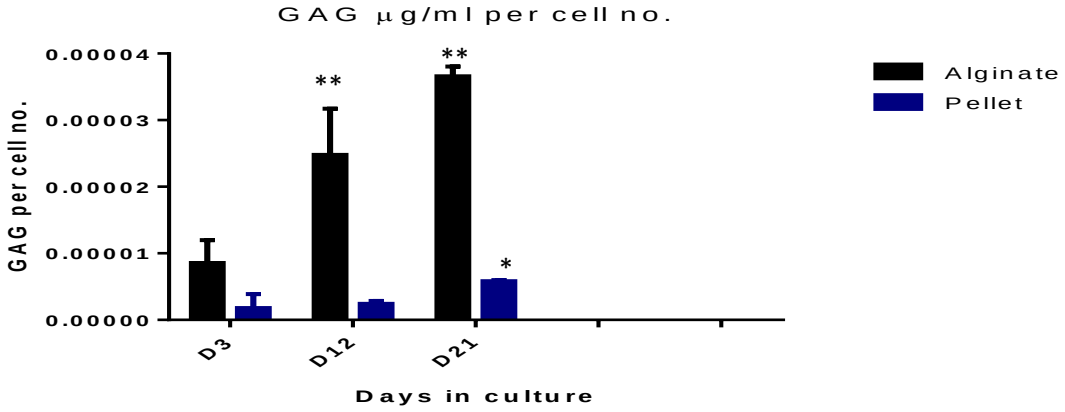


Figure 3-8 Biochemical analysis of sulphated GAGs content

Cumulative GAG content of supernatant (µg/ml) in alginate and pellet culture after reduction of background GAG, Mean ± SD. * $p<0.05$, ** $p\leq0.01$

3.4.3 Proliferation assay

Comparison between proliferation of chondrogenic experimental groups showed that only in alginate beads cell proliferation was increased overtime at day 21 as compared to day 3 ($p=0.05$), while in pellet culture cell proliferation remained the same at all time-points ($p>0.05$). In monolayer cell number significantly reduced overtime (Fig. 3-8 B; $p<0.05$).

In 3D cultures of pellet and alginate control (non-induced) groups, cell proliferation decreased overtime on day 21 compared to day 3, but increased in 2D monolayer (Fig. 3-8 A; $p=0.05$).

In all experimental groups, there was no significant increase in the percentage reduction of AlamarBlue between day 0 and day 3, in monolayer group the values were higher but not significant ($p>0.05$). From day 3 to day 12 only monolayer control cultures showed higher cell proliferation ($p=0.05$) and in pellet control there was a significant decrease in the value (Fig. 3-8 A; $p=0.05$). In chondrogenic groups there was no significant increase in the percentage of reduction of AlamarBlue (Fig. 3-8 B; $p>0.05$).

Comparison between day 12 and day 21 in chondrogenic groups showed significant increase in cell proliferation in alginate group and decrease in monolayer ($p=0.05$). There was no significant increase in chondrogenic pellet culture ($p>0.05$). In control groups, cell proliferation in alginate group decreased significantly ($p=0.05$) but there was no significant different in monolayer and pellet culture groups ($p>0.05$).

Comparison between groups using Kruskal Wallis test showed no significant difference between cell proliferation in all chondrogneic groups except for day 21 that alginate group was significantly higher than pellet and monolayer (Fig. 3-8 B; $p<0.05$). Comparison between groups showed no significant different in cell proliferation on day 0 ($p>0.05$).

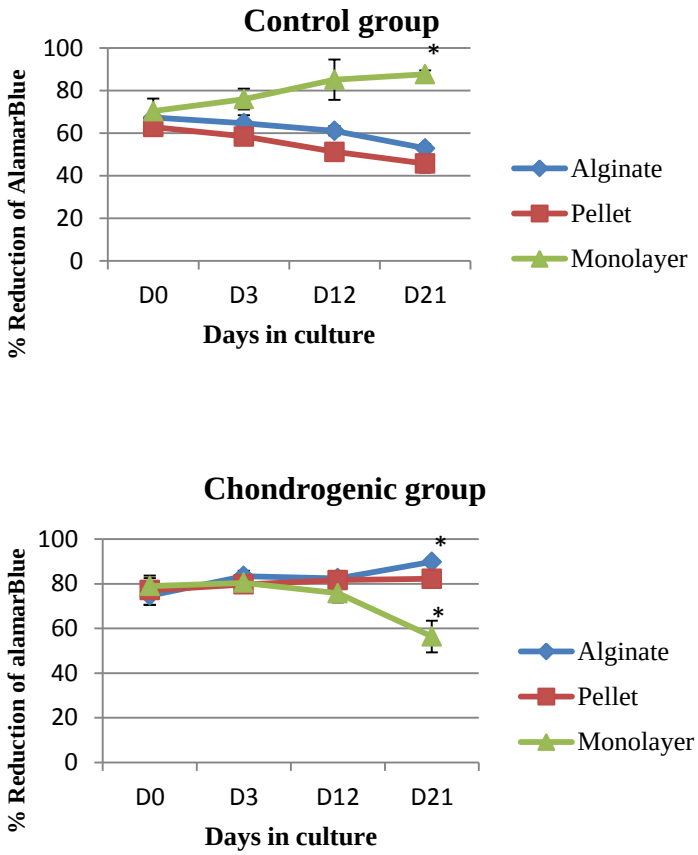


Figure 3-9 Percentage reduction of AlamarBlue in experimental groups in different time points

Percentage reduction of AlamarBlue A. Control groups B. Chondrogenic groups. Mean ±SD, *p≤0.05.

3.4.4 Live/Dead cell assay

Qualitative test of cell viability using Calcein/EthD-1 is shown in (Fig. 3-9) for different experimental groups on day 3 and day 21. In chondrogenic alginate group cell viability was higher on day 21 when it compared with day 21 control.

Chondrogenic pellet culture showed a solid spherical shape on day 21 using normal fluorescent microscopy, cell viability was inconclusive in a packed texture (Fig. 3-9 F), but in control group cells were dispersed and cell death visibly increased on day 21 when it compared with day 3 (Fig. 3-9 G&H). In chondrogenic monolayer detached patches of cells in each medium change was an indication of high cell death in this group. Although staining with EthD did not show any red spots due to the removal of dead cells during media change and washing with PBS before staining, empty spaces between cells on day 21 implied cell death (Fig. 3-9 J) when it compared with the control group (Fig. 3-9 L).

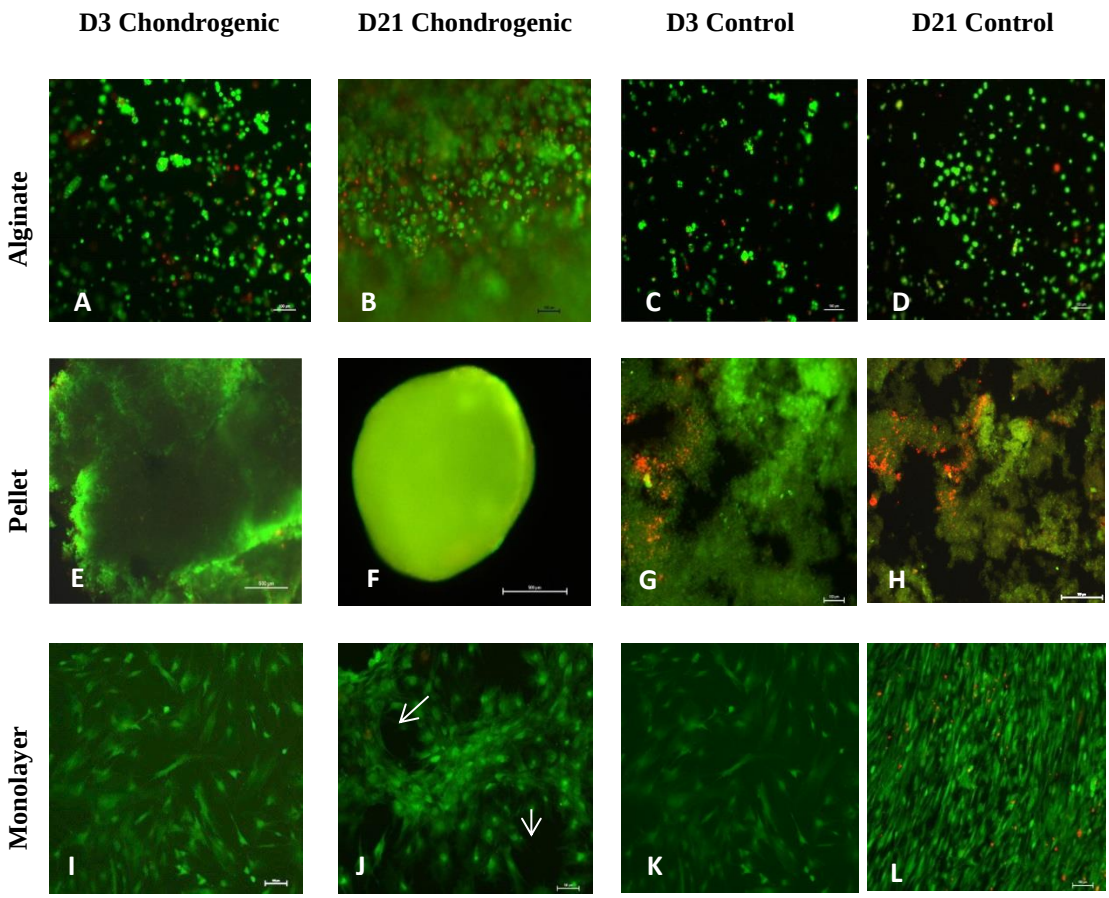


Figure 3-10 Live/Dead cell staining using Calcein/EthD-1

Green colour indicates live cells, red spots show dead cells, Alginate 10X, pellet chondrogenic 4X, control 10X, Monolayer 10X. Arrows indicate empty spaces resulted from detached cells.

3.4.5 Scanning Electron Microscopy (SEM)

MSCs surface was studied using SEM before and after chondrogenic differentiation. In an undifferentiated MSCs (day 0) cell surface covered with protrusions, filopodia or blebs (Fig. 3-10 A&B).

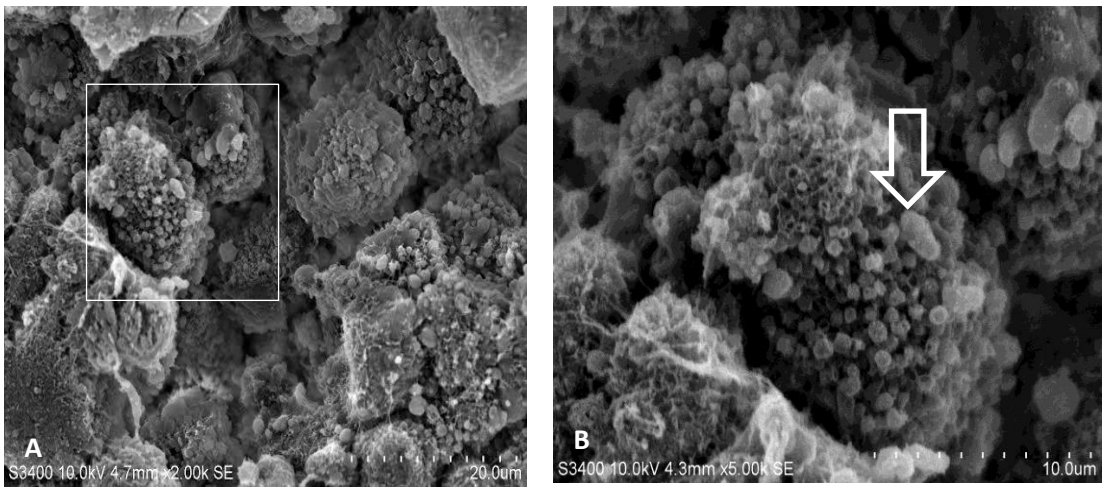


Figure 3-11 SEM image of MSCs day 0

A. Human MSC day 0 2000X.
B. Higher magnification of the inset at A. 5000X the surface is covered with protrusions (filopodia/blebs) (open arrow).

Cell surface in chondrogenic differentiated MSCs in alginate were covered in extracellular matrix (ECM), while layers of ECM were deposited between the cells (Fig. 3-11).

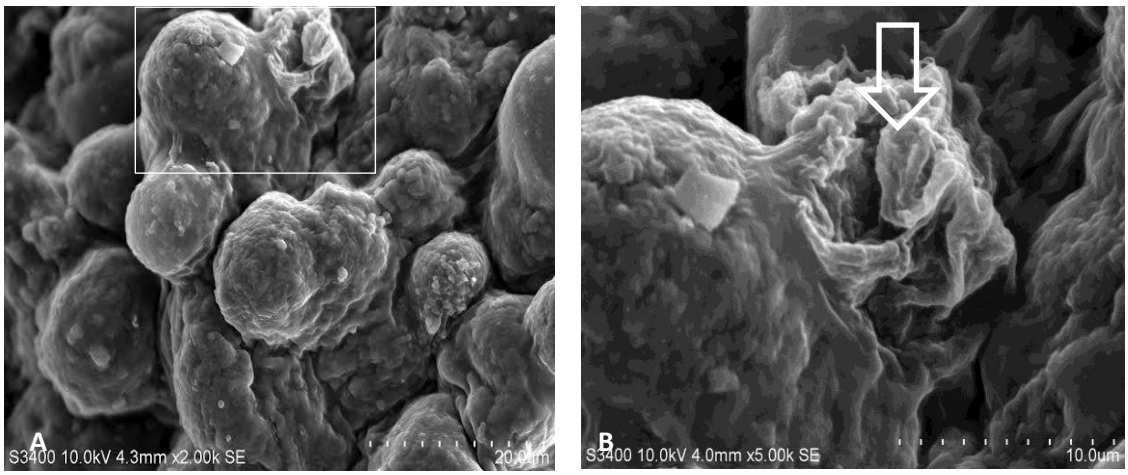


Figure 3-12 SEM image of chondrogenic alginate group day 21

A. Chondrogenic MSC in alginate beads day 21 2000X.
B. Higher magnification of the inset 5000X shows cell surfaces covered with ECM. ECM deposited between the cells (arrow).

3.4.6 Transmission Electron Microscopy (TEM)

Resin embedded samples of chondrogenic pellet and alginate on day 21 as well as MSCs on day 0, were cut into 1µm semi thin sections, stained with Toluidine Blue and

studied under light microscopy (Fig. 3-12). Ultrathin sections of 70nm thickness on copper grids, stained with uranyl acetate and lead citrate studied with electron microscopy (Fig. 3-13 to 3-20).

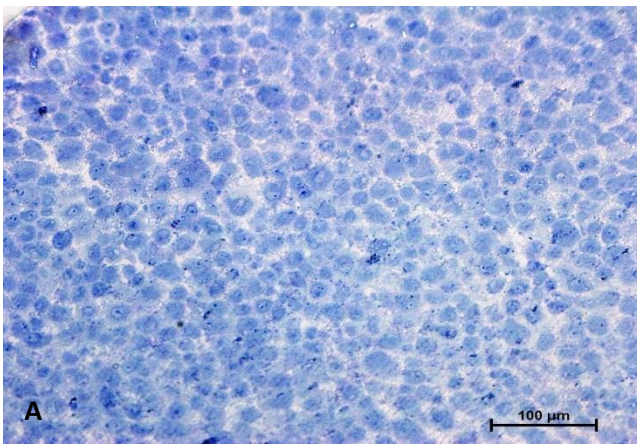
3.4.6.1 Semi-thin sections

The prominent feature of semi-thin sections of alginate beads compared to pellet cultures was a lower cellularity, and increased inter-cellular spaces (Fig. 3-12 B double head arrow). In alginate cells arranged in small groups of two or three cells and the matrix between them stained purple using toluidine blue (Fig. 3-12 B arrow). While in pellet culture cells were in closer contact with each other and less purple colour indicated lower extra cellular deposition (Fig. 3-12 C).

The presence of abundance poly anions in the extracellular matrix of cartilage gives a purple colour to the metachromatic dyes such as Toluidine Blue (Sridharan G. *et al.* 2012). Samples of MSC day 0 as a control group stained blue, samples of CMSC in alginate and pellet culture showed positive metachromatic areas (purple colour), especially in alginate group in the matrix surrounding the cells (Fig. 3-12 B arrow) an indication of GAG deposition.

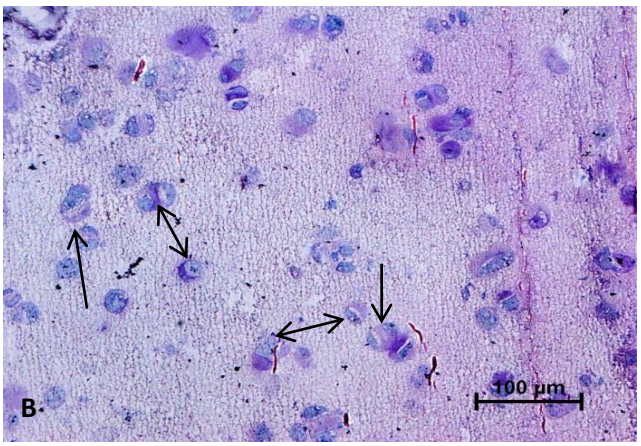
Undifferentiated

MSC day 0



CMSC day 21

Alginate culture



CMSC day 21

Pellet culture

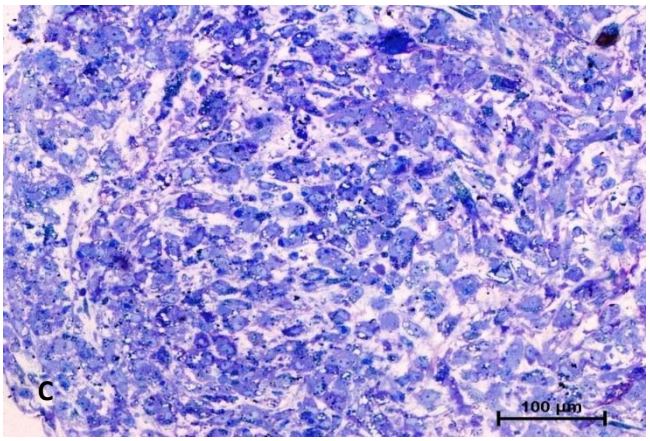


Figure 3-13 Semi-thin sections of chondrogenic alginate and pellet day 21

A. MSC day 0.
B. CMSC in alginate bead day 21.
C. CMSC in pellet culture day 21 Semi-thin section 1µm 20X Toluidine Blue staining.

3.4.6.2 Ultrathin Sections

MSCs: Cell surface of MSCs formed filopodia or blebs (Charras G. *et al.* 2008) associated with dens bodies (Fig. 3-13 B&C). Cytoplasm occupied with rER, free

ribosomes, mitochondria (M), and vacuoles (Fig. 3-13). Nuclei were euchromatin, usually with a distinct nucleolus, demonstrating an active protein synthesizing cell, in some cells the nucleus was convoluted (Fricker M. *et al.* 1997, Fig. 3-13A).

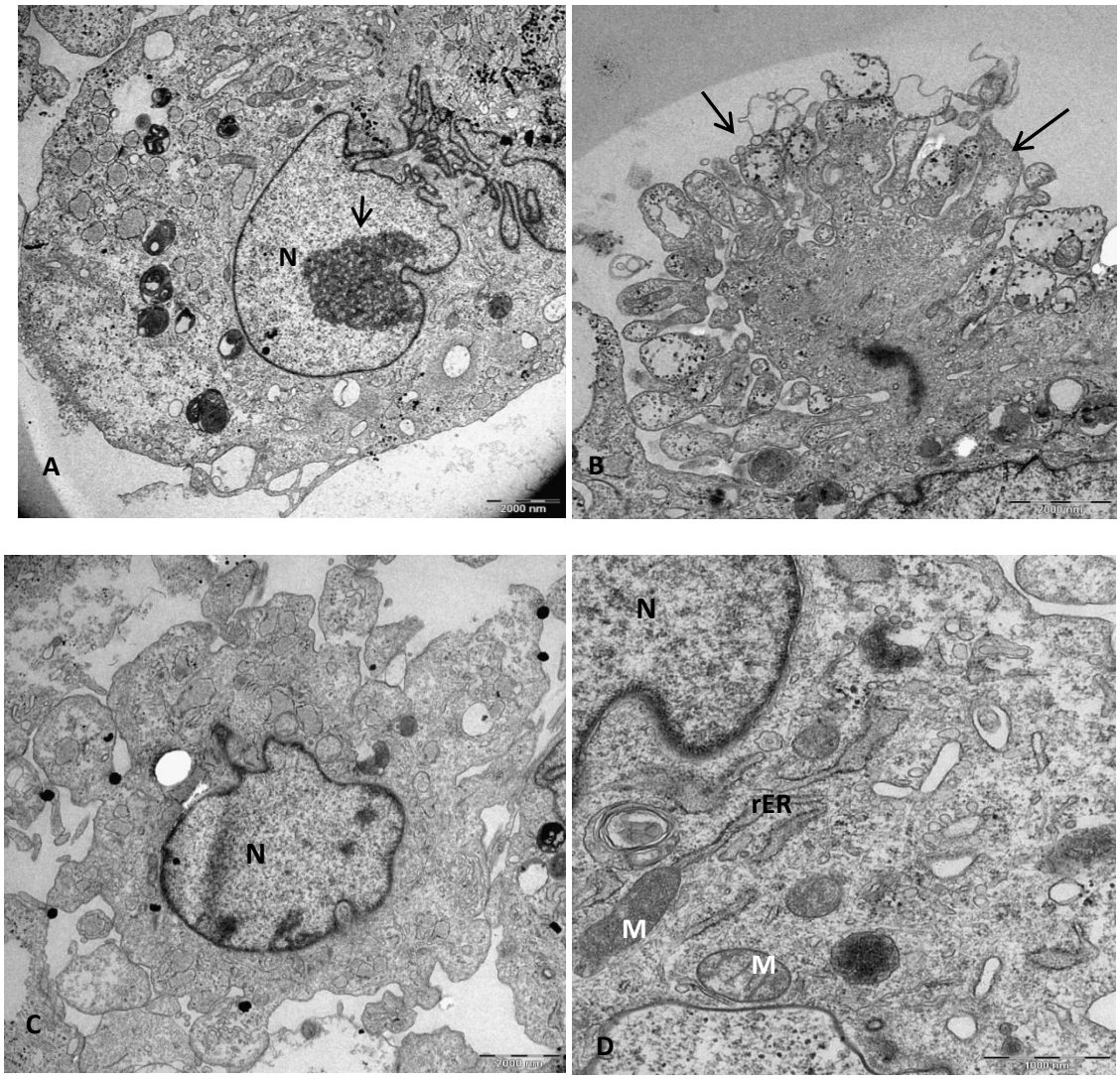


Figure 3-14 TEM images of MSCs day 0

A. MSC with a convoluted nucleus and prominent nucleoli (arrow) 1151X.
B. Blebs in the cell surface (arrow) 1600X N=Nucleus.
C. MSC with a round nucleus and uneven cell surface 1233X.
D. Higher magnification of MSCs shows mitochondria (M) and rough endoplasmic reticulum (rER) 4000X.

In chondrogenic groups of alginate and pellet the cells actively produced extracellular matrices, as it was evidenced with a highly developed rER full of secretions, fibrils and fibres in the cytoplasm and in the ECM (Fig. 3-14, 3-15, 3-16, 3-17, 3-19).

Alginate: Ultrastructure of nucleus in CMSC showed euchromatin with variety of phenotype, round, oval or slightly indented (Fig.3-14 & 3-17).

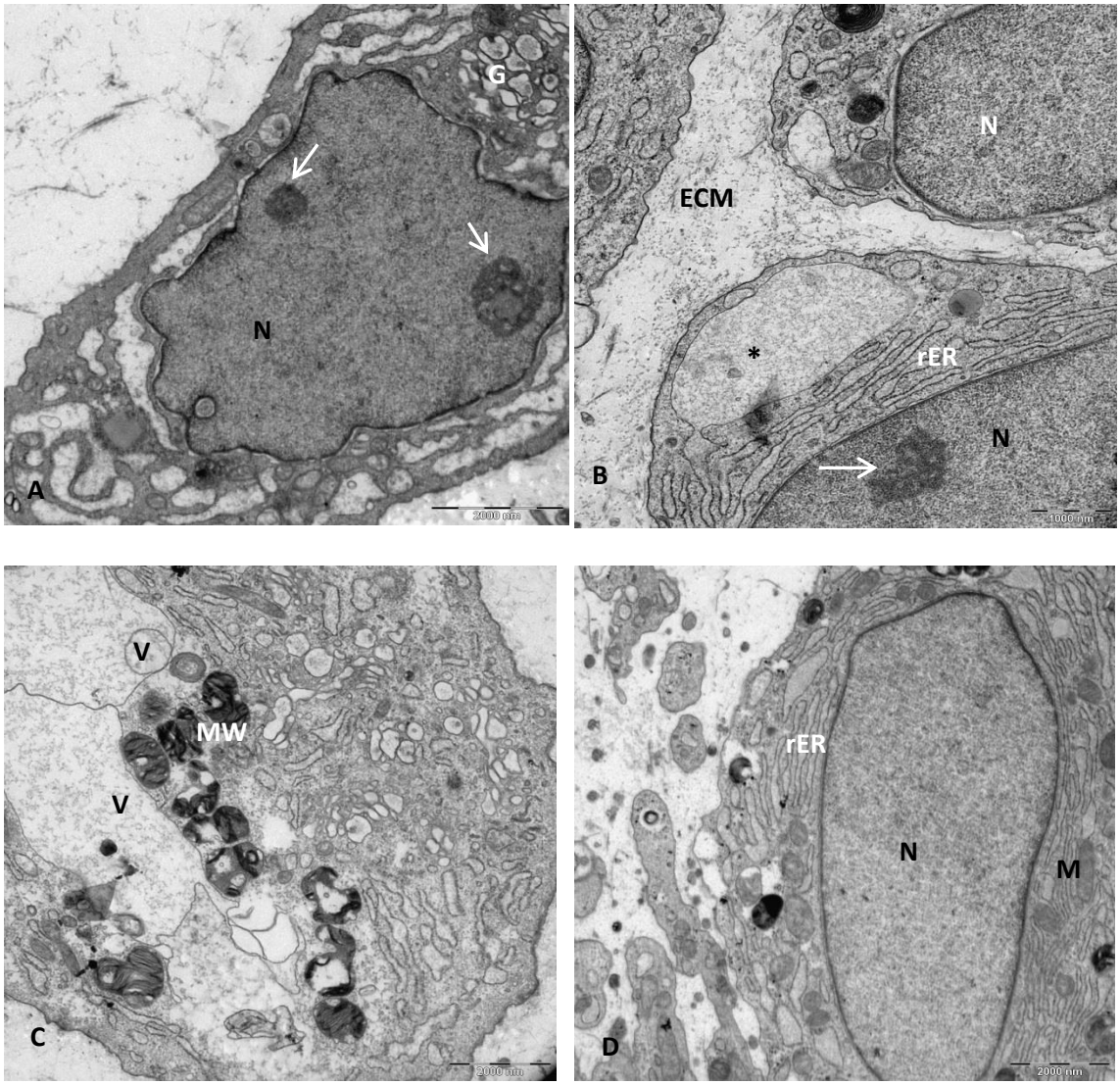


Figure 3-15 TEM images of chondrogenic alginate group day 21

Different cell morphology in CMSC-alginate, N=Nucleus, RER=Rough endoplasmic reticulum.

A. The cytoplasm is filled with distended RER, Golgi apparatus in the supra-nuclear region (G) 2000X.
B. Three cells similar to isogenic groups in cartilage deposit their products in the ECM=Extracellular matrix 2520X.
C. A cell undergoing cell death with no clear nucleus and abundant cytoplasmic vesicles (V) and cytoplasmic inclusions or multilayer whorled membrane (MW) 1575X.
D. A cell with a euchormatic nucleus, rER and mitochondria (M) 1575X.

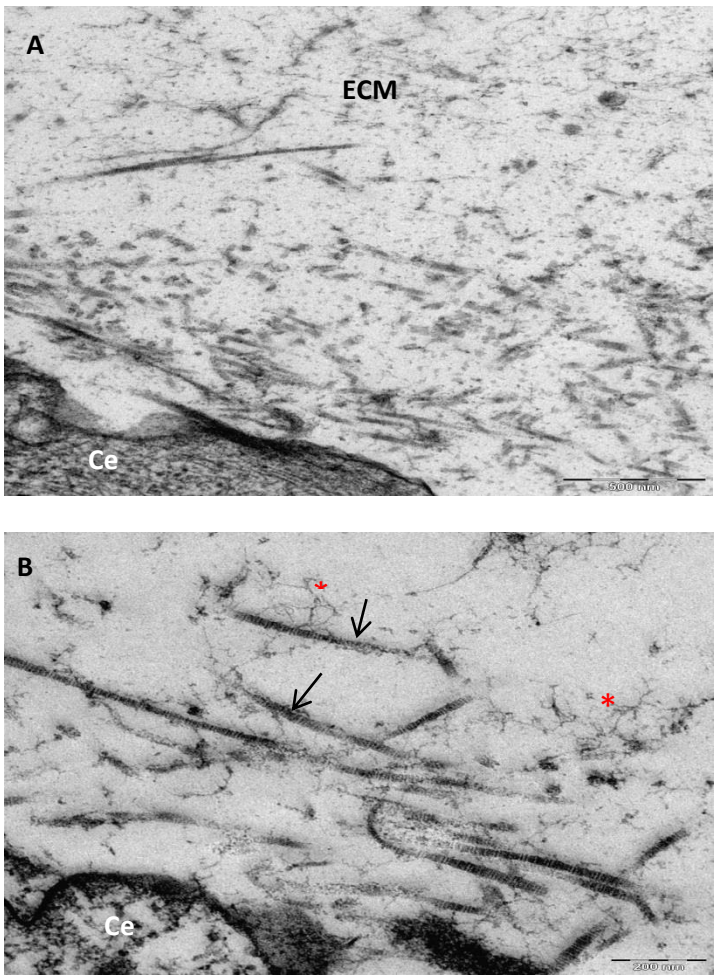


Figure 3-16 TEM images of ECM in alginate group

A. Territorial matrix surrounding CMSC. ECM=Extracellular matrix, Ce=cell 6300X.
B. Higher magnification shows striation of collagen fibers (arrow) and branched molecules probably aggrecan (asterisk) 10000X.

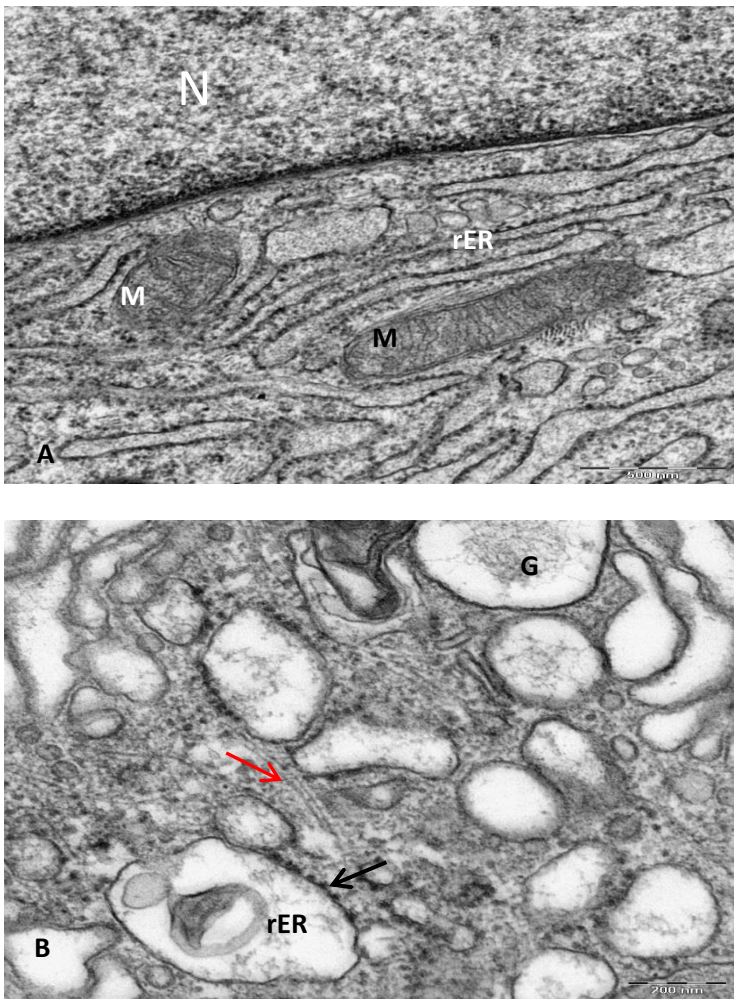


Figure 3-17 TEM image of perinuclear cytosol in chondrogenic alginate group

A. A part of nucleus (N) and cytoplasm containing rough endoplasmic reticulum (rER) and Mitochondria (M) 6300X.
B. High magnification of cytoplasm. G= Golgi, black arrow indicates ribosomes, Red arrow shows microtubules, rER= Rough endoplasmic cytoplasm 10000X.

Chondrogenic MSCs in alginate showed characteristic features of cells that are actively involved in protein synthesizing (Pavelka M., *et al.* 2010), with euchromatic nuclei, prominent nucleoli, abundant rER filled with electron lucent materials, producing abundant ECM, containing collagen fibres (Fig. 3-14, 3-15, 3-17) and molecules with side branches similar to proteoglycan aggregates among the fibres (Fig. 3-15B). Chondrogenic differentiated cells usually arranged in small groups as it was seen in lower magnification in semi thin (Fig. 3-12B) and ultrathin sections (Fig. 3-14B & 3-17A). Higher magnification of adjacent cells did not show any junctional complexes

between the cells. Smaller vesicles contained electron lucent material near cell membrane (Fig. 3-17 A) might originate from rER releasing their content to ECM, causing the cells grow apart from each other, similar to that seen during the interstitial growth in cartilage.

In highly active protein synthesizing, chondrogenic cell in alginate in which the cytoplasm was packed with rER, it was difficult to distinguish between distended rER and Golgi apparatus (Fig. 3-14 A), however in higher magnification the rER can be easily recognized with studded ribosomes (Fig. 3-16 B) (Pavelka M., *et al*. 2010)

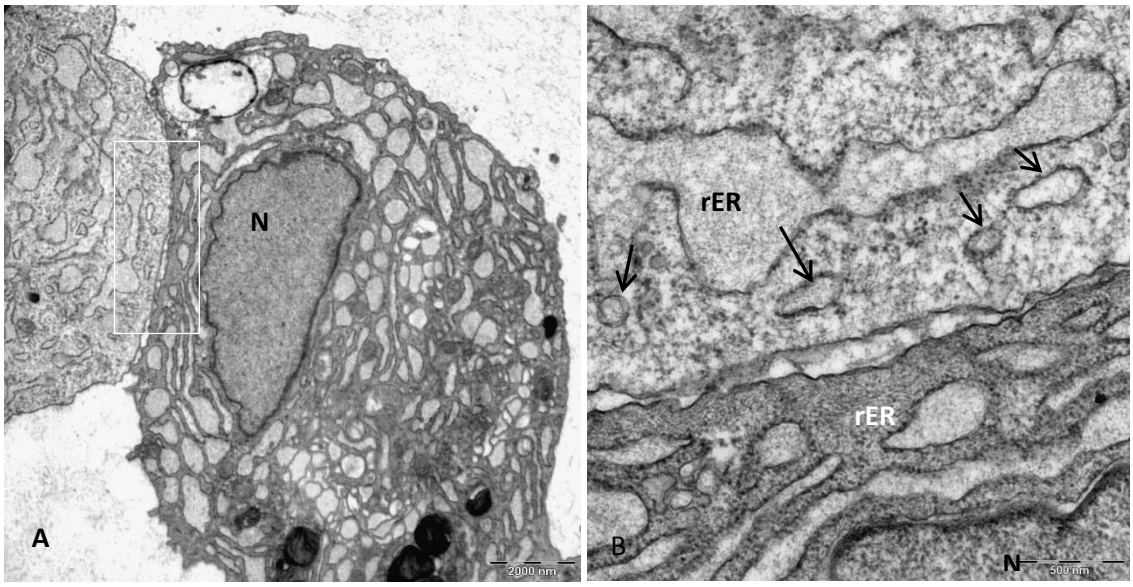


Figure 3-18 TEM image of divided cells in chondrogenic alginate group

A. Two daughter cells resulted of cell division 1260X.
B. Higher magnification of the inset shows ECM (asterisk) in the inter- cellular space. Note small vesicles near cytoplasmic membrane (arrows), rER=rough endoplasmic reticulum 6300X.

Pellet culture: In general, two subpopulation of cells could be distinguished in ultrathin sections of pellet culture: In one group the signs of cell death appeared (Kumar V., *et al.* 2013) with abundant cytoplasmic vesicles, lipid droplets, free ribosomes, swelled or fused mitochondria (Fig. 3-18 A), vacuoles, expelling of cytoplasmic organelles to ECM (Fig. 3-18 B), indented nucleus (Fig. 3-18 A&D) or cells without a prominent

nucleus (Fig. 3-18 C). The second population consisted of active protein synthesizing cells with a euchromatin and round nucleus, abundance of collagen fibres secreted in the ECM (Fig. 3-19 & 3-20). Golgi apparatus can be observed in both groups (Fig. 3-18 D & Fig. 3-19 A) as machinery for synthesis of carbohydrate (Alberts B. *et al.* 2002) or GAG in chondrogenic induced MSCs.

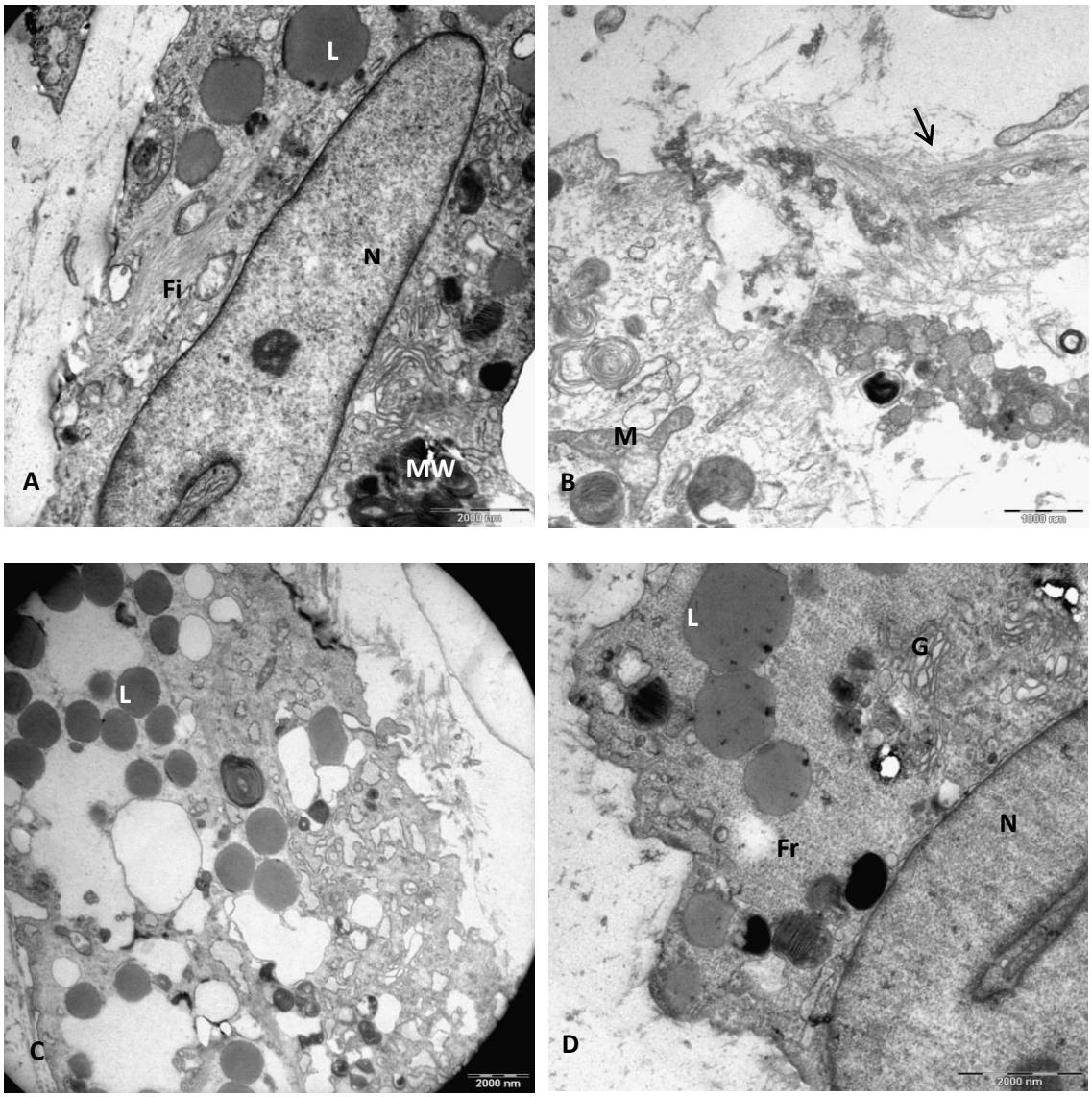


Figure 3-19 TEM images of cell death in chondrogenic pellet culture

A. Increase of lipid droplet, N=nucleus MW= Multivesicular membrane, L=lipid droplet, M=mitochondria, Fi=cytoplasmic fibrils, 1600X.
B. Fused mitochondria (M), expelled cell organelles including mitochondria (arrow) 2520X.
C. No clear nucleus 1000X.
D. Cell with U-shape nucleus, free ribosomes (FR) in cytoplasm, Golgi apparatus (G), and lipid droplets (L) 1984X.

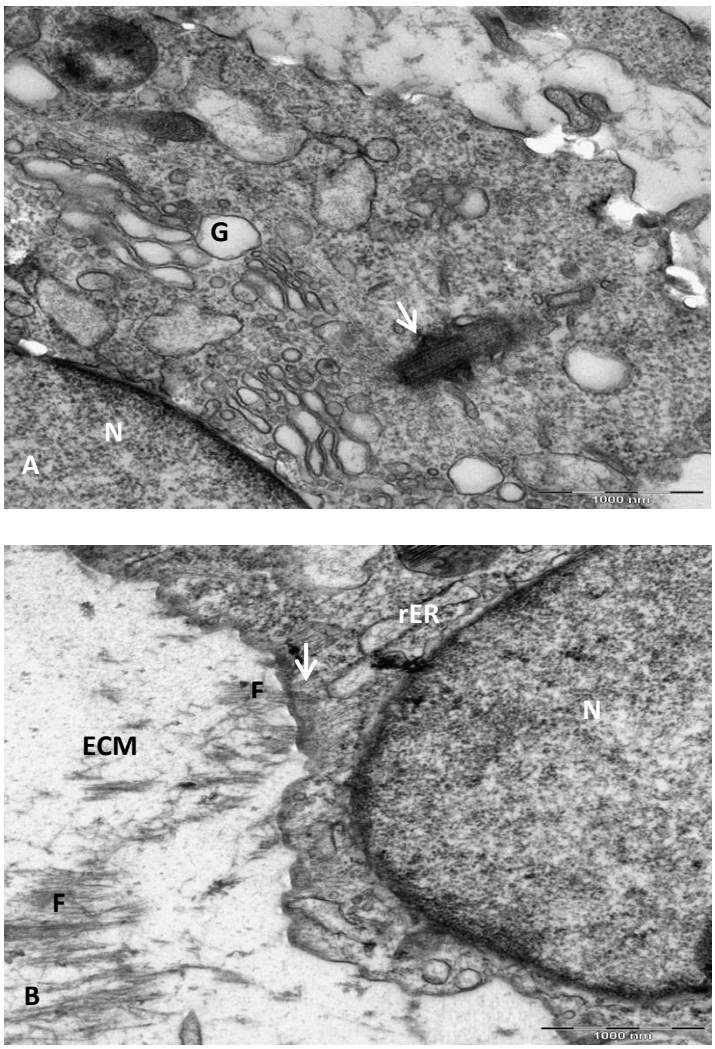


Figure 3-20 TEM image of chondrogenic pellet day 21

A. Well-defined Golgi apparatus and Centriole (arrow) in the perinuclear cytosol 4000X.
B. Collagen fibers (F) secreted to the ECM directly from the cytoplasm (white arrow) 3969X.

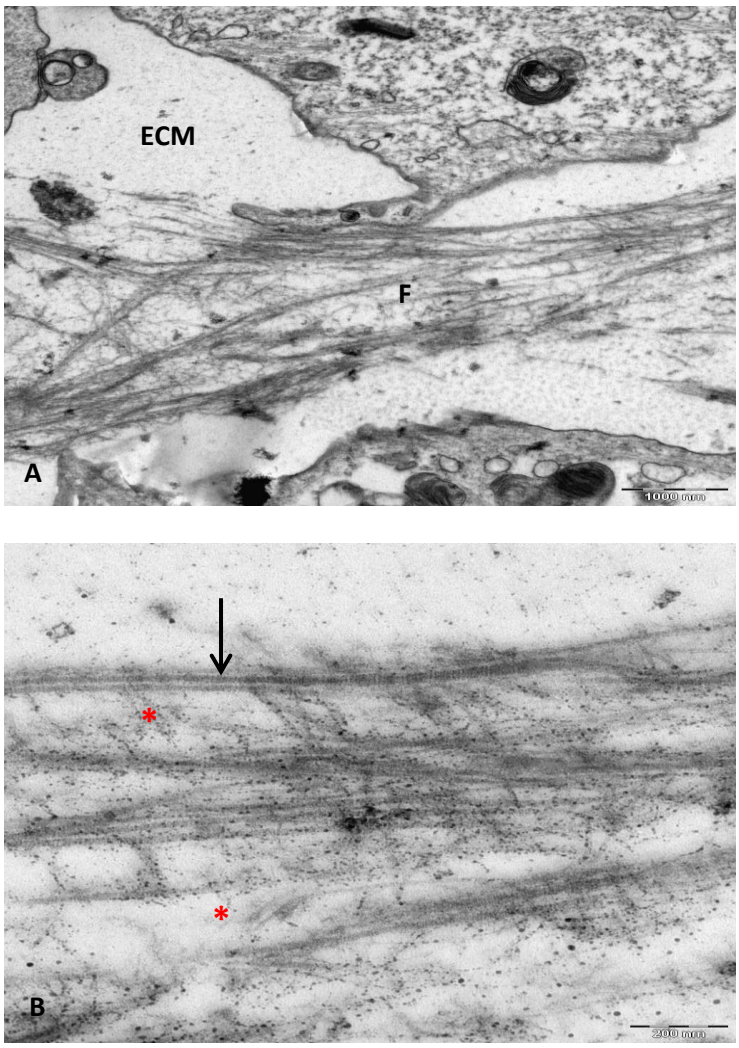


Figure 3-21 TEM image of ECM in chondrogenic pellet day 21

A. Collagen fibers (F) in the ECM 2500X.
B. Higher magnification 10000X shows striation (arrow) on fibers. Small molecules (black dots) and branched molecules between the fibers (asterisk).

3.5 Discussion

In this study, superior production of GAGs demonstrated in CMSC alginate group during chondrogenic differentiation compared to chondrogenic pellet culture and monolayer and control groups, using Safranin O Fast green staining (Fig. 3-2 & 3-3) and quantitative GAGs analysis (Fig. 3-7). It was shown that (Derfoul A. 2007) glucosamine (GlcN), a chemical constitute of GAGs, promotes chondrogenesis in human MSCs and chondrocytes phenotype while inhibiting the expression of MMP-13. Alginate with a structure simulating GAGs may have such an effect on MSCs chondrogenesis, in addition to providing a spherical morphology and isotropic cytoskeletal tension to MSCs (Nava M.M. *et al* 2012) a prerequisite for chondrogenic differentiation (Guilak F. *et al.* 2009).

Moreover, higher expression of GAGs during chondrogenic differentiation of MSCs was shown to be associated with increase in shear moduli of the constructs (Awad H.A. *et al.* 2004). In this study, although the mechanical properties of alginate constructs were not tested, higher expression of GAGs may be an indirect indication of higher biomechanical moduli of alginate constructs after chondrogenic differentiation. This property may make CMSC alginate constructs a superior candidate for cartilage repair application compared to undifferentiated MSCs in alginate.

Immunohistochemistry for chondrogenic markers collagen II and aggrecan were consistent with GAG result. However the positive results of immunohistochemistry for collagen I can be an indication of undifferentiated or fibroblastic like cells among the chondrogneic differentiated groups. In fact, among the chondrogenic groups, collagen type I was the highest expressed protein in 2D monolayer accompanied with a hypertrophic morphology (Fig. 3-6). This may indicate a premature hypertrophy of the chondrogenic induced MSCs in monolayer. It also showed that in 2D chondrogneic monolayer, although Col II expressed weakly on day 21 (Fig. 3-4C), GAG and ACAN,

the important ECM compartments of cartilage, were only expressed in a 3D environment of pellet and alginate cultures.

Alizarin Red staining showed no deposition of minerals during chondrogneic differentiation of MSCs in 3D cultures of pellet and alginate, while in another study (Ichinose S. *et al.* 2005), Alizarin Red positive calcification was reported during chondrogenic differentiation of MSC in alginate beads in a defined medium containing TGFβ3. During endochondral ossification of large bones, the primary cartilage template replaces with bone, which is as a result of removing the hypertrophic chondrocytes by osteoclasts and mineral deposition by osteoblasts brought to the ossification centres by blood vessels under regulation of endothelial growth factor (VEGF) isoforms (Yang Y.Q. *et al.* 2012). In previous literature, the possibility of transdifferentiation of hypertrophic chondrocyte to osteoblast remained to be proved in subsequent studies, however based on the study on hypertrophic chondrocytes in epiphyseal plate it suggested that the local environment may have a role in determining cell fate during endochondral ossification (Adams C. S. *et al.* 2002).

Among the chondrogenic groups, alginate showed the highest cell proliferation and monolayer showed the lowest proliferation rate. Pellet culture did not show any significant changes in cell number during chondrogenic differentiation. These results were consistent with viability tests using Calcein/EthD. In lung bud organ culture and prostate epithelium it was shown that TGFβ had an inhibitory effect on cell proliferation, but in mammary epithelia it had a bi-functional role in carcinogenesis, as during neoplastic transformation the growth inhibitory role changed to stimulatory effect (Moses H.L. *et al.* 1994). TGFβ was shown to increase cell proliferation and invasiveness of rat placental cell line as well (Lafontaine L. *et al.* 2010). In our study chondrogenic MSCs in alginate beads under defined medium containing TGFβ3 exhibited a higher proliferation rate compared to the same cells in pellet and monolayer

cultures. It seems that the inhibitory or stimulatory effect of TGFβ3 on MSCs proliferation depends on the 3D structure and biochemical properties of the microenvironment.

MSCs loaded in alginate beads in growth medium, did not show any cellular proliferation during the experiment. In such condition, adhesive dependent MSCs remain in a quiescent phase, since they do not receive any signals through adhesion molecules in a suspension-like alginate. Although adherent cells undergo apoptosis or Anoikis if they do not have correct cell/ECM attachment in suspension (Gilmore A.P. *et al.* 2005), in a viscous medium such as synovial fluid of rheumatoid arthritis patient, MSCs survived (Swart J. *et al.* 2008). It is possible that with a similar mechanism, MSCs may be able to survive in a viscous alginate culture system. In a chondrogenic medium, MSCs produce chondrogenic ECM and have an interaction with the newly formed ECM probably via integrins and CD44 (Lee G.M. *et al.* 1998). They later proliferate through the downstream signalling pathways (Schwartz M.A. *et al.* 2001; Walker J.L *et al.* 2005). In our experiment cell proliferation in chondrogenic alginate group only was observed on third week following chondrogenic differentiation and expression of chondrogenic protein markers, as it was shown by immunohistochemistry or biochemical analysis of collagen type II, ACAN, and GAG.

Actin remodelling at the MSCs surface produces protrusions or projections surrounding the cells or construct (Fig. 3-10). Formation of actin based protrusions including filopodia and lamellopodia or blebs has been shown to be linked with the migratory and invasiveness potential of the cells (Fackler O.T. *et al.* 2008). While in chondrogenic differentiated MSCs, the alginate at the cell surface were embedded in a thick ECM (Fig. 3-11).

TEM study revealed an increase in cell death in pellet culture, as compared to alginate culture. It can be due to an immature hypertrophy and cell death, as it may happen during endochondral ossification (Adams. C. S. *et al.* 2002; Mackie E.J.*et al.* 2008). The type of cell death can be non-apoptotic or physiologic cell death, since it lacked the whole characterization of apoptotic cells such as crescent heterochromatin nucleus (Zamli Z. *et al.* 2011). However, there is a possibility that distinctive morphology of apoptotic nucleus is missing in our experiment due to its occurrence in an earlier time point.

The morphology and abundance of mitochondria in non-induced MSCs and alginate culture were similar (Fig. 3-13 & 3-16). Hypoxic microenvironment in cartilage may have caused lower number of mitochondria in articular cartilage compared to metabolically active cells since chondrocytes rely on glycolytic metabolism rather than oxidative phosphorylation (Milner P.I. *et al.* 2012). In this study we did not perform any quantitative method for comparing mitochondria in MSC and CMSC, however the qualitative pictures did not show any differences in the mitochondrial density between non-induced MSCs and chondrogenic MSCs, it might be the culture condition with ambient oxygen 21% that do not similar to normal habitat of chondrocyte in cartilage with low oxygen concentration 2-10% (Zhou S. *et al.* 2004) it has been shown chondrocytes isolated from the joint and cultured *in vitro* expressed mitochondrial biosynthesis (Milner P.I. *et al.* 2012). Further studies using immunofluorescent for detecting mitochondria can verify mitochondrial quantities during *in vitro* chondrogneic differentiation of MSC.

In pellet culture, swollen and fused mitochondria may be due to hypertrophy and cell death. Mitochondrial fusion was shown in cells undergoing cell death. Fusion of mitochondria is described as a reaction of the cells to damaged mitochondria to repair by inter-mixing DNA and protein between mitochondria during damage or senescence

(Chan D.C. *et al.* 2006). Swollen mitochondria was also reported in fibrillated cartilage in OA patients (Roy S. *et al.* 1968).

The morphology of the nuclei in the chondrogenic groups of alginate and pellet were varied. These appeared more elongated and indented in pellet, whilst in alginate it appeared mostly oval and spherical. Although a typical chondrocyte is observed with a spherical nucleus, previous ultrastructureal studies have shown variations in shapes including oval, spherical, elongated or indented in human articular cartilage, which appears to be acceptable for chondrocyte morhpology (Roy S. *et al.* 1968).

3.6 Conclusion

The results demonstrated superior chondrogenesis in 3D cultures compared to 2D monolayer and superior chondrogenesis in alginate compared to pellet cultures in chondrogenic medium. MSCs in different experimental groups cultured in growth medium did not demonstrate chondrogenic activities, therefore suggesting that alginate alone *in vitro,* cannot induce chondrogenesis of MSCs without the presence of chondrogenic stimulating factors such as TGFβ. Detailed ultrastructural observations using TEM supported histological and biochemical quantitative results. Although 'live/dead' staining was inconclusive in pellet cultures, TEM images revealed cell death prevalence in pellet culture compared to alginate culture.

4 CHAPTER4

Study 2: Chondrogenic, hypertrophic and adhesion molecule gene expression during chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells in alginate beads, pellet culture, and monolayer

4.1 Study design

In this part of the study human bone marrow derived mesenchymal stromal cells (MSCs), were isolated, expanded until passage 3 and characterized with criteria (Dominici M. *et al*. 2006) such as tri- lineage differentiation and expression or lack of expression of specific CD markers using immunocytochemistry and flow cytometry. The cells were then divided and cultured into two groups: one group with chondrogenic medium and the other group with growth medium. Each group contains cultures of monolayer, alginate beads and pellet culture. In different time points (day 3, day 12 and day 21) RNA was extracted from different groups and converted to cDNA. Gene expression was studied using Real Time Polymerase Chain Reaction (RT-PCR) for chondrogenic markers such as Sox9, Collagen type II, aggrecan; hypertrophic markers included RunX2 and Collagen type X. Adhesion molecules such as NCAM1 and N-Cadherin and collagen type I as a non-chondrogenic gene.

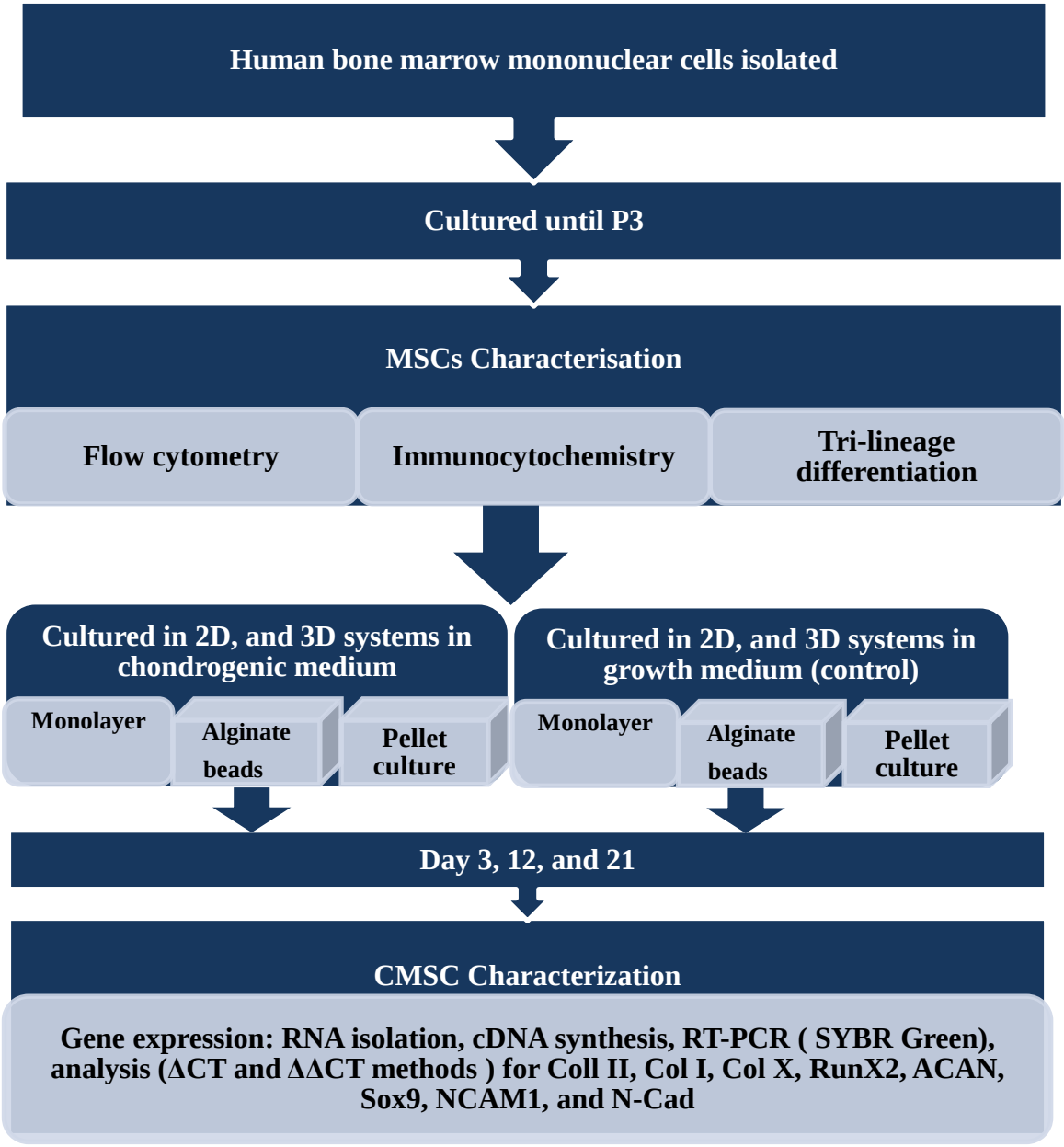


Figure 4-1 Flowchart shows the study design for chapter 4.

4.2 Introduction

Chondrogenic differentiation is a unique process that initiates with cell-cell interactions, and requires the influence of specific growth and differentiation factors. During embryonic limb formation this process is described as condensation. Based on this phenomena different chondrogenic induction techniques have been developed to recapitulate the *in vivo* chondrogenesis. In such methods usually high density of cells in shape of pellet or aggregates or a combination of cells with biomaterials are applied to provide a three-Dimentional (3D) structure for maximum cell-cell and cell-matrix interactions (Penick K.J. *et al.* 2005). Integrins a heterodimer receptor responsible for cell-matrix and cell-cell interactions, have been studied extensively during chondrogenesis *in vitro* and *in vivo* (Shakibaei M. 1998; Fukumoto T. *et al.* 2002; Goessler U.R. *et al.* 2006; Lu Z.F. *et al.* 2008).

Expression of N-CAM and N-cadherin as two major adhesion molecules has been shown during condensation of MSCs in limb bud formation (Monroy J.C. *et al.* 1999; Woodward W.A. *et al.* 1999; Hall B.K. *et al.* 2000). These molecules were later down-regulated after chondrogenic differentiation and only expressed in periphery of the limb anlagen *in vivo* or chondrogenic aggregate *in vitro* (Widelitz R.B. *et al.* 1993) (Tavella S *et al.* 1994). In articular cartilage, each chondrocytes individually is responsible for production of extracellular matrix (ECM) as functional unit of cartilage or chondron, (Poole C.A. *et al.* 1997) and there is no direct cell-cell contact between chondrocytes (Mobasheri A. *et al.* 2009). In this study gene expression of N-CAM1 and N-Cadherin was explored in different chondrogenic models of Alginate bead, Pellet, and monolayer cultures. We assumed that a chondrogenic differentiation model that has the least expression of cell-cell adhesion molecules along with the high expression of chondrogenic markers was more similar to articular cartilage.

The comparison of 2D and 3D culture systems conventionally has been used to study chondrocyte dedifferentiation *in vitro* (Domm C. *et al.* 2002, Caron M.M. *et al.* 2012). However, a systematic investigation into the role of 2D and 3D cultures with or without alginate as a scaffold to demonstrate the potential for chondrogenic differentiation of MSCs had not been performed at the time this study was conducted. In this study, 2D monolayers were used as the control group to support the rationale of using 3D cultures (Pellet and alginate) for chondrogenic differentiation of MSCs; while pellet culture was used as a known hypertrophic model to be compared with an alginate chondrogenic model of MSCs.

4.3 Methods and Materials

4.3.1 Isolation of human bone marrow stromal cells

Healthy human bone marrow samples (male, age=21$\pm$2.6 years) from patients undergoing long bone fixation, were used for gene expression. This study was approved by the University of Malaya Medical Centre Ethics Committee (Reference no. 472.95). Human bone marrow was collected in sterile 3ml BD Vacutainer blood tubes (K2 EDTA, BD franklin Lakes NJ USA) by orthopaedic surgeons and was kept at 4°C until isolation. The mononuclear cells were isolated using Ficoll density gradient method as described in chapter 2, cultured and expanded until P3 in 75ml culture flasks. The cells were confirmed as MSCs by characterization through flow cytometry using cell surface markers (positive and negative markers) and their ability to undergo tri-lineage differentiation (adipogenic, chondrogenic and osteogenic differentiation) as described in chapter 2.

4.3.2 Chondrogenic differentiation and experimental groups

4.3.2.1 Pellet culture

MSCs were harvested at P3, 250X 10^3 cells were pelleted in a 15ml propylene centrifuge tube, at 1100 rpm for 5 minutes. After removal of the supernatant, the pellet cultured either with 2ml of basal growth medium containing 10% FBS or chondrogenic medium containing: DMEM high glucose (4.5mg/ml D-Glucose) with sodium pyruvate (110 µg/ml) (invitrogen). ITS Sigma 50mg/ml(1X) (invitrogen), L Ascorbate 2 phosphate (50µg/ml) Sigma, TGFβ3 10ng/ml (invitrogen), Dexamethason 100nM ($1x10^{-7}$M) (Sigma), Penicillin/Streptomycin 100µg/ml (invitrogen), L Proline 40µg/ml (Sigma), or growth medium containing high glucose DMEM supplemented with 10% FBS and 1% pencillin/Streptomycin. The media were changed every 3 days.

4.3.2.2 Cell-alginate constructs

1.2% alginate prepared from alginic acid powder, low viscosity (Sigma-Aldrich) in 0.9% sodium chloride (NaCl) and filtered sterile by a 0.2µm filter.

MSCs at P3 were harvested and a concentration of $4X10^6$ cell per ml of alginate were obtained before dropping into sterile, calcium chloride solution ($CaCl_2$) using a pipette. Alginate bead constructs cross linked in this solution for 10 minutes in 37°C incubator and then were rinsed in 0.9% normal saline 2-3 times, and transferred to the culture dishes (ultra-low attachment 12 well plates, Corning). Three beads per well (about 80 000 cells per bead) and supplemented with 2ml of chondrogenic or growth media. The medium changed each 3 days.

Alginate cells constructs were dissociated in a buffer solution contained 0.015M sodium citrate and 0.15M sodium chloride ($Na_3C_6H_5O_7$ dehydrate $2H_2O$, MW=294.10) pH 7.2, centrifuged at 1100 rpm for 5 minutes before gene expression studies were conducted.

4.3.2.3 Monolayer culture

In two groups P3 cells were cultured in chondrogenic or growth media at density of 4000 cells/cm^2 in 6 well plates.

4.3.3 Gene expression studies (real time PCR)

4.3.3.1 Primers

Human gene sequences were obtained from NCBI gene bank the primers were designed using Primer 3 software and NCBI primer designing tool (Table 4-1). Annealing temperature of 58.6 was chosen for primers after being tested by gradient PCR using RT-PCR CSFXTM 96.

4.3.3.2 Positive control

Human normal chondrocyte (Clonetics™ normal human articular chondrocytes (*NHAC-kn*) Lonza, Walkersville. Inc. U.S.), embedded in alginate beads for 21 day was used as a positive control for gene expression. The data were normalized to βactin expression while MSCs day 0 considered as a calibrator in ΔΔCt formula.

4.3.3.3 RNA isolation

RNA was isolated from cell pellets using SV total RNA isolation system, Promega (USA) according to the manufacturer instructions. Chondrogenic groups of pellet and alginate bead after dissociation with sodium citrate buffer, on day 12 and day 21were digested with 300-500µl of 0.1% solution of collagenase type II (Worthington, Lakewood NJ.) in PBS (1.5 hours in 5% CO_2, 37°C incubator) before RNA isolation**,** then centrifuged at 200g for 5 minutes after adding 1X PBS to make pellets**.**

The quality and purity of RNA was measured using a NanoDrop 2000 (ThermoScientific, UK) spectrophotometer. The integrity of RNA was determined using gel electrophoresis for 28S and 18S ribosomal RNA. The ratio of 2/1 for 28S/18S and the sharpness of the bands (without smears) were considered intact RNA. The RNA was kept in -80°C until further processing to cDNA.

4.3.3.4 cDNA synthesis

In each reaction 100ng RNA were converted to first strand cDNA in 20µl final volume using iScript™ Reversed Transcription Supermix for RT-qPCR (BioRad) according to the manufactures' protocol. No template controls (NTC) were used in each reaction as negative control.

4.3.3.5 Quantitative Polymerase Chain Reaction

PCR reactions were carried out in duplicate for each biological samples (N=3) in a final volume of 20µl containing SYBR Green mastermix (BioRad) with 160nM concentration of each primers and 200ng cDNA in 96-well plates (Bio-Rad) using CSFX96™ Real Time System, Bio-Rad under the following conditions: 3.1 minutes at 95°C followed by 40 cyles of 58.6°C for 0.20 minutes as annealing temperature and 72°C for 0.30 minutes as extension. The reactions were ended with 0.1 minutes of 95°C and a melt curve by increasing temperature from 65°C, 0.05 minutes to 95°C, 0.5 minutes stepwise.

The data were presented as a time fold change relative to the internal control gene expression. The data then normalized to transcription levels of day 0 culture using ΔCT and ΔΔCT methods (Livak K.J. *et al.* 2001). Values below 1 were considered down regulated. The following primer sets were applied in this experiment:

Table 4-1 Primer sequence

Gene	Access no.		Primer pairs 5'-3'	Amplicon Size
Aggecan	NM_001135.3	F	CTACGACGCCATCTGCTACA	141
		R	TCAGTGATGTTTCGAGGCAG	
Beta-actin	NM_001101.3	F	CTCTTCCAGCCTTCCTTCCT	116
		R	AGCACTGTGTTGGCGTACAG	
Collagen I	NM_000088.3	F	ACCTGGTCAAACTGGTCCTG	122
		R	CCTGTGGTCCAACAACTCCT	
Collagen II	NM_033150.2	F	GAAAGCCTGGTGATGATGGT	138
		R	GGCCTGGATAACCTCTGTGA	
Collagen X	NG_008032.1	F	CACCTGTGGTCCTGAATGTG	163
		R	TCTGAGTGCCTGGATGTCTG	
N-Cadherin	NM_001792.3	F	GGAAAAGTGGCAAGTGGCAG	159
		R	GGAGGGATGACCCAGTCTCT	
NCAM1	NM_000615.6	F	AGGAGACAGAAACGAAGCCA	161
		R	GGTGTTGGAAATGCTCTGGT	
Run X2	NM_001015051.3	F	TTACTTACACCCCGCCAGTC	139
		R	CACTCTGGCTTTGGGAAGAG	
SOX9	NM_000346.3	F	AGACAGCCCCCTATCGACTT	108
		R	CGGCAGGTACTGGTCAAACT	
GAPDH	(Bian L. et al., 2011)	F	AGGGCTGCTTTTAACTCTGGTAAA	NA
		R	GAATTTGCCATGGGTGGAAT	

4.3.4 Statistical analysis

The different between experimental groups was calculated using non-parametric Kruskal-Wallis H test, and the different between two independent experimental groups using Mann-Whitney U test, available on the statistical software package SPSS (version 18.0) with $p \leq 0.05$ was being considered significant.

4.4 Results

4.4.1 Gene expression in human chondrocyte

The prominent genes expressed in human chondrocytes were collagen type II, type X, and aggrecan (ACAN). Collagen type I, cell adhesion molecules NCAM1 and N-Cadherin were down-regulated. Sox9, slightly up regulated and the expression of RunX2 gene was not significant (Fig. 4.1).

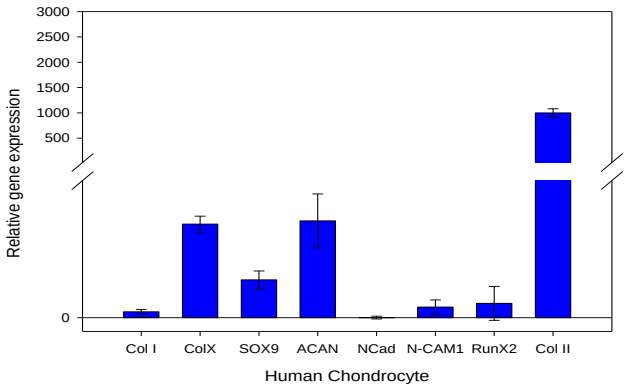


Figure 4-2 Gene expression in human chondrocytes as positive control

4.4.2 Gene expression in experimental groups

4.4.2.1 Collagen type II

Comparison of chondrogenic samples using Mann-Whitney test showed significantly higher expression of Col II in alginate group compared to pellet and monolayer culture ($p=0.05$). comparison between pellet and monolayer showed no difference in Col II expression on day 3 ,but over time, pellet culture showed a higher expression of col II on day 12 and 21 compared to monolayer culture ($p=0.05$).

Expression of collagen II in all experimental groups were significantly increased between day 3 and day 12 ($p\leq0.05$), but from day 12 to day 21 the increase was not significant in alginate group ($P>0.05$) and in monolayer it decreased non-significantly

(p>0.05). However in pellet culture the increasing trend from day 12 to day 21 was significant (p<0.05). In general in all experimental groups expression of collagen II increased significantly from day 3 to day 21 (P≤0.05) (Fig. 4-2).

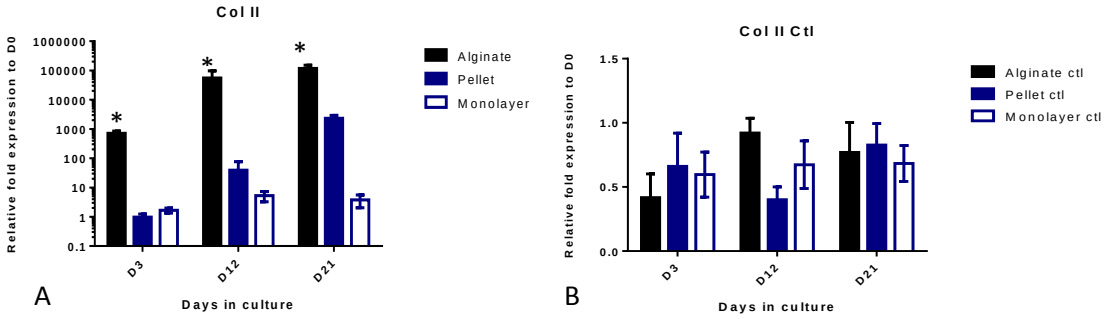


Figure 4-3 Gene expression of Collagen II

A. Chondrogenic groups B. Control groups * p≤0.05.

4.4.2.2 Sox9

Comparison between group in chondrogenic groups showed significant differences between Sox9 expression in alginate group with pellet and monolayer cultures on day 12 and day 21 (p=0.05), while there was no difference between groups on day 3 (p>0.05). Sox9 expression significantly increased from day 3 to day 12 (p=0.05) in alginate group but the increase from day 12 to day 21 was insignificant (p>0.05). These comparisons were insignificant in monolayer culture for all time points. In pellet culture the different of Sox9 expression between day 3 and day12 was insignificant (p>0.05) but between day 3 and day 21 was significant (p=0.05) (Fig. 4-3).

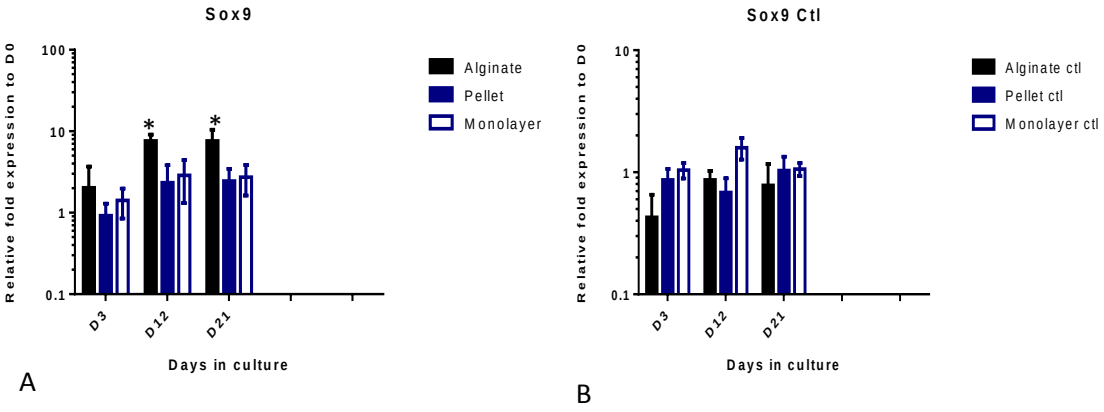


Figure 4-4 Gene expression of Sox9

A. chondrogenic groups B. Control groups *p≤0.05.

4.4.2.3 Aggrecan (ACAN)

ACAN gene expression in alginate group was significantly higher than pellet culture in all time points (P<0.05), whereas in monolayer group no ACAN expression was observed. In alginate group the expression of ACAN increased significantly from day 3 to day 12 (p=0.05) but in another two groups there were no significant increase in these time points (p>0.05). In alginate, ACAN expression did not increased from day 12 to 21 (p>0.05), but in pellet culture it significantly increased (p<0.05). In both groups of alginate bead and pellet cultures ACAN gene expression increased from day 3 to day 21 significantly (p=0.05) (Fig. 4-4).

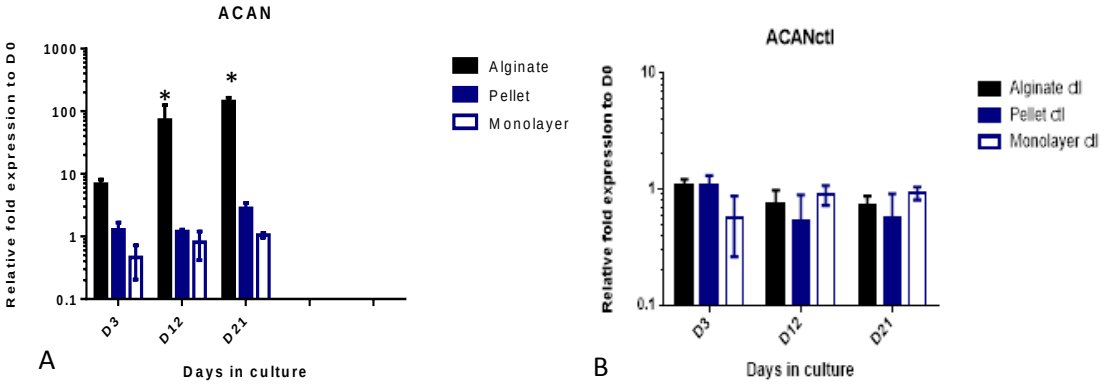


Figure 4-5 Gene expression of ACAN

A. Chondrogenic groups B. Control groups *p≤0.05.

4.4.2.4 Collagen type I

Gene expression of Col I were significantly different among the chondrogenic experimental groups on different time points using Kruskal-Wallis test (P<0.05).

The different was especially significant between time points in pellet culture group in which it significantly increased on day 21 (P<0.05). Comparison between two groups using Mann-Whitney test showed that expression of Collagen I was higher in alginate bead compared to pellet and monolayer culture at all-time points (P=0.05). The comparison between monolayer and alginate primarily showed higher expression of Collagen I in monolayer on day 3 (p=0.05) but there was no significant different between two groups on day 12 and day 21 (p>0.05).

Col I expressed higher on day 12 compared to day 3 in alginate bead but in other two groups the increase in col I was not significant (p>0.05). However in pellet culture it was significantly higher on day 21 compare to day 12 (p=0.05) while in alginate and monolayer culture groups the differences at this time points were not significant (p>0.05). In general in all experimental groups col I significantly increased from day 3 to day 21 (Fig. 4-5; P=0.05).

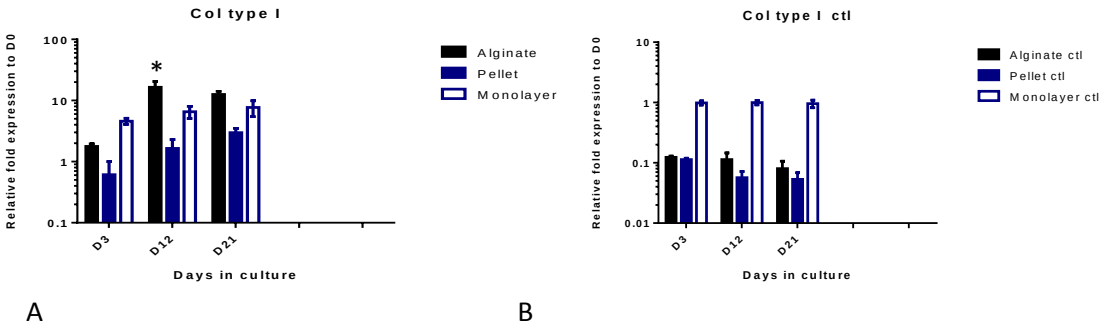


Figure 4-6 Gene expression of Collagen I

A. Chondrogenic groups B. Control groups *p≤0.05.

Table 4-2 Ratio of Collagen I to Collagen II gene expression

Groups	Col I	Col II	Col I/Col II
Alginate	12.5	116900	0.000107
pellet	3	2300	0.001304
Monolayer	7.6	3.8	2

Data represents fold expression of genes in chondrogenic groups compared to day 0 MSCs

4.4.2.5 Collagen type X

Comparison between chondrogenic groups using Kruskal-Wallis test showed the highest expression of collagen X in monolayer group on day 3 and day 12, while pellet culture showed the highest expression of this gene on day 21. The expression of Collagen X in alginate group was significant ($p<0.05$) among different time points with the highest on day3 but down regulated on day 12 and day 21. In pellet and monolayer the differences were insignificant ($p>0.05$). Pellet culture expressed the highest level of Col X expression on day 21 and monolayer on day12. Mann-Whittny test showed the difference in the expression of Col X between alginate group and pellet on day 3 and day 21 was significant ($p=0.05$). On day 3 alginate had higher expression while on day 21 on contrary pellet culture showed higher expression than alginate. The differences in the expression of Col X on day 12 was not significant between two groups of pellet and alginate cultures ($p>0.05$).

Comparison between pellet culture and monolayer showed Col X significantly expressed higher in monolayer on day 3 and day 12 ($p=0.05$), however on day 21 the different was not significant between two groups ($p>0.05$). The expression of Col X in alginate group compared to monolayer on day 3 was not significant ($p>0.05$), but over time on day 12 and day 21 monolayer expressed significantly higher amount of Col X (Fig. 4-6; $p<0.05$).

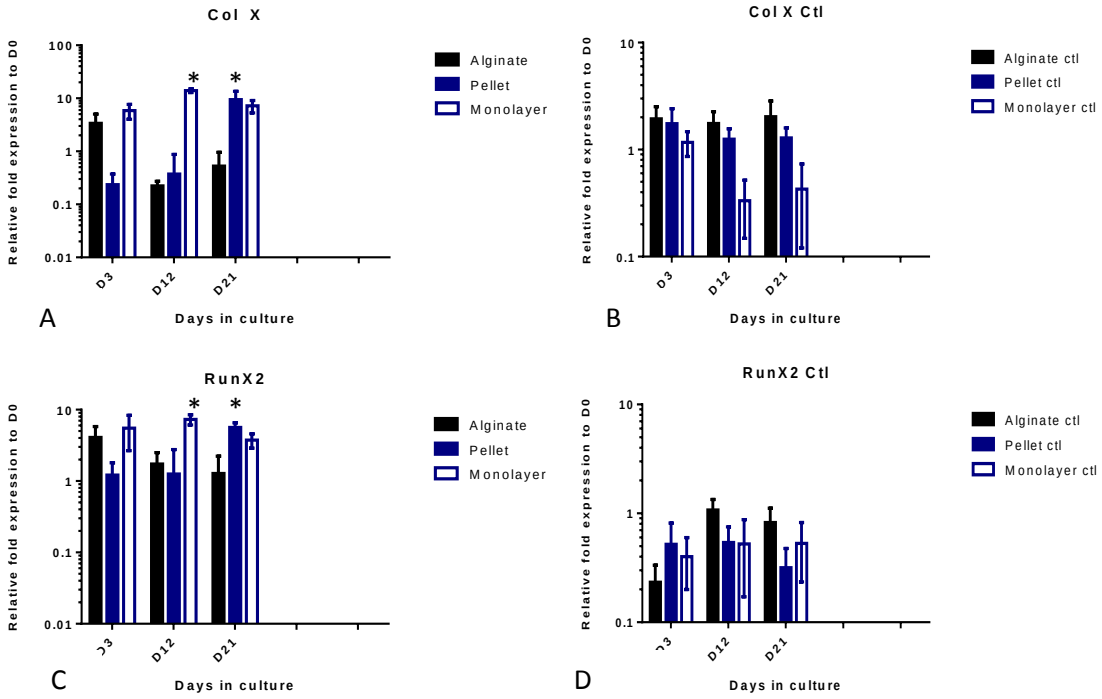


Figure 4-7 Gene expression of Collagen X and Run X2

A & C. Chondrogenic groups. B & D. Control groups *p≤0.05.

4.4.2.6 Run X2

Gene expression differences of RunX2 on day 3 and day 21 was significant between alginate and pellet culture (p=0.05), on day 3 alginate group had a higher expression but on day 21 RunX2 was more expressed in pellet culture. There was no statistically significant different between the two mentioned groups on day 12 (p>0.05).

Comparison between pellet and monolayer culture using Mann-Whitney test showed higher expression of RunX2 on day 3 and day 12 in monolayer than pellet culture. However on day 21 expression of RunX2 was more prominent in pellet than monolayer culture (p=0.05). Comparison between alginate and monolayer on day 3 showed no significant difference between the two groups (p>0.05), but on day 12 and day 21

expression of RunX2 was significantly higher in monolayer than alginate culture ($p=0.05$).

In alginate group, expression of RunX2 decreased significantly from day 3 to day 12 ($p=0.05$) but the difference on day 12 compared to day 21 was insignificant ($p>0.05$). In monolayer and pellet culture the difference in the RunX2 gene expression was not significant from day 3 to day 12 ($p>0.05$), but in monolayer it decreased and in pellet increased significantly on day 21 ($p=0.05$).

In general in alginate and monolayer culture RunX2 expression decreased overtime. The difference in alginate group was significant between time points over time ($p=0.05$) but in monolayer was insignificant ($p>0.05$). In pellet culture expression of RunX2 was significantly increased over time (Fig. 4-6; $p<0.05$).

4.4.2.7 N-CAM1

N-CAM1 remained down-regulated on day 3 and day 12 during chondrogenic differentiation of MSCs in pellet culture and up regulated on day 21, but in alginate group it up-regulated over time from day 3 to day 21. In monolayer culture the expression of N-CAM1 in chondrogenic group increased significantly from day 3 to day 12 ($P<0.05$) and then down-regulated from day 12 to day 21 ($p<0.05$).

Expression of NCAM1 in CMSC in alginate group increased over time from day 3 to day 21 ($p=0.05$) but the increase in Pellet culture was not significant (Fig. 4-7; $p>0.05$).

4.4.2.8 N-Cadherin (N-Cad)

N-Cad was down-regulated in pellet culture and alginate group on day 3 and day 12 but it was up-regulated on day 21. In CMSC alginate culture N-Cad increased significantly from day 3 to day12 ($p=0.05$) but in another two experimental groups the increase was not significant ($p>0.05$). N-Cad increased in pellet culture from day 12 to day 21 significantly ($p=0.05$) but the increase of the gene expression in alginate culture was not significant ($p>0.05$) and it decreased in monolayer culture though not significantly ($p>0.05$).

Comparison between pellet and monolayer showed N-Cad significantly expressed higher in monolayer than pellet culture on day 3 and day 12 ($p=0.05$). However on day 21 it was less expressed in monolayer than pellet culture ($p=0.05$). On day 3 chondrogenic monolayer showed higher expression of N-Cad compared to alginate culture but there was no significant differences on day 12 and day 21 between these two groups (Fig. 4-7; $p>0.05$).

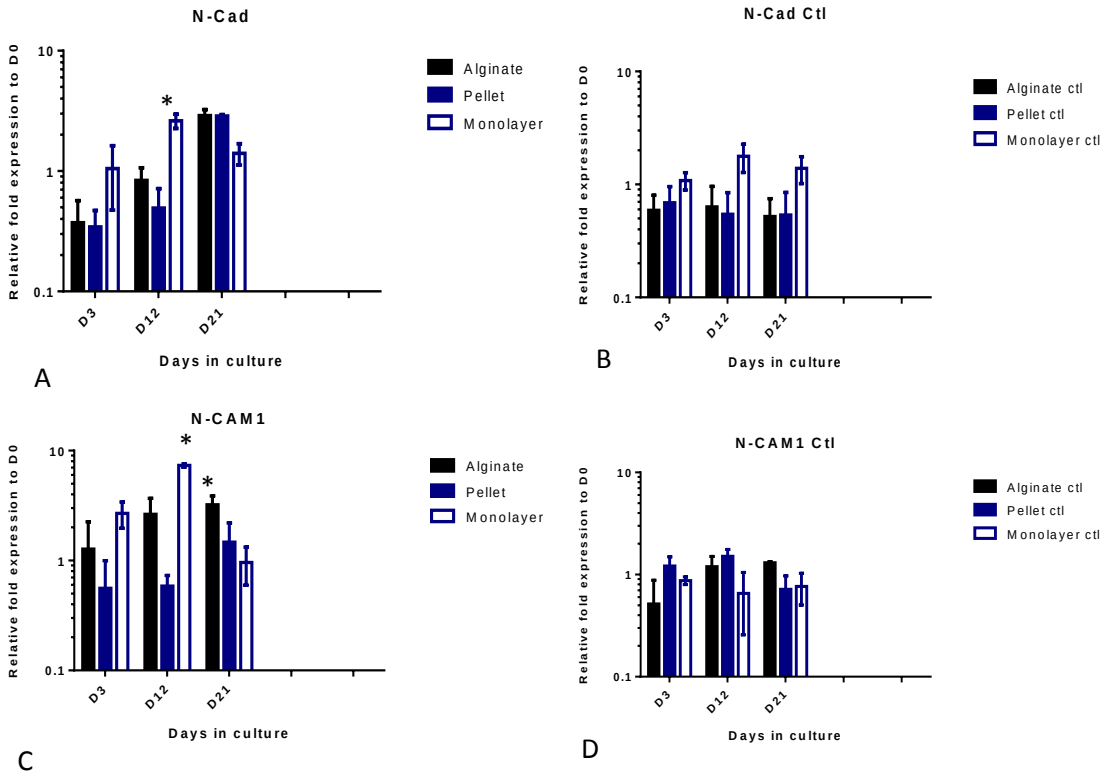


Figure 4-8 Gene expression of N-CAM1 and N-Cad.

A & C: Chondrogenic groups. B. & D. Control groups *p≤0.05.

4.5 Discussion

Gene expression of cartilaginous markers which includes collagen type II and ACAN in CMSC alginate culture in this experiment was higher than that of mature normal human chondrocytes. This is due to the fact that newly formed chondrogenic construct in alginate bead resembles an immature foetal cartilage in which anabolic activity surpluses the catabolic activities in chondrocytes and results a higher production of ECM compared to an adult cartilage tissue (Aigner T. *et al.* 2006).

Gene expression results showed superior chondrognenic differentiation of MSCs in 3D culture of alginate compared to pellet culture and 2D culture of monolayer. These results are consistent with previous researches (Yang I.H. *et al.* 2004) but it is different from Ichinose S. *et al.* 2005 in which during chondrogenic differentiation of MSCs in

alginate beads induced with TGFβ3 calcification was reported. In our study hypertrophic genes of RunX2 and Col X down regulated/did not expressed in alginate group, but they up-regulated in pellet and monolayer culture under similar culture medium.

Expression of transcription factor Sox9 is accompanied by the expression of chondrogenic genes, such as Col II, and ACAN (de Crombrugghe B. *et al.* 2000). In this study the increase of gene expression of Sox9 overlaps with the expression of Col II and ACAN genes in 3D chondrogenic systems as well, however in chondrogenic monolayer it was not accompanied with an increase of ACAN. In this experiment expression of Sox9 remained low in monolayer and chondrognic pellet culture model, while the expression of Col X and Run X2 were prominent in these groups, considering the fact that Sox9 inhibits hypertrophic changes in chondrocytes (Bi W. *et al.* 1999, Murakami S. *et al.* 2004; Ikegami D. *et al.* 2011; Dy P. *et al.* 2012), in this study higher expression of Sox9 in alginate culture may have protected the cells from being differentiated to a hypertrophic conditions.

Comparison between chondrogenic and control groups showed an increase in the expression of collagen type I in chondrogenic groups. The increase synthesis of collagen type I in fibroblasts has been shown by TGFβ3 through Smad3 protein, *in vitro* and *in vivo* (Verrecchia F. *et al.* 2007). It might be a possibility that a fraction of cells transdifferentiated to fibroblast/myofibroblasts during chondrogenic differentiation and produce col I under the influence of TGFβ3.

Expression of collagen type I was also shown in other researches during chondrogenic differentiation of bone marrow derived MSC in pellet or alginate culture (Kosher R.A. *et al.*1986; Barry F. *et al.* 2001; Endres M. 2009; Jang M.Y. *et al.* 2013, Fernandes A.M.,. *et al.* 2013). It seems that current static chondrogenic induction conditions have

yet to be optimized, and by applying mechanical loading in a bioreactor, culture condition may be improved and enhances chondrogenesis by reducing collagen type I formation (O'Conor C.J. *et al.* 2013). In our study although expression of Collagen I in alginate beads was higher than pellet and monolayer culture. The ratio of Collagen I to Collagen II in alginate group was lowest among the groups, while in chondrogenic monolayer culture the expression of Col type I dominated the expression of collagen type II (Table 4-2).

In our study, cell adhesion molecules of N-CAM1 and N-Cad were down regulated in 3D cultures of alginate beads and pellet cultures on day 3. These results are consistent with down regulation of these genes during embryonic chondrogenesis (Widelitz R.B. *et al.* 1993; Tavella S. *et al.* 1994), in which N-CAM1 and N-Cad down regulated after expression of chondrocyte specific genes. On the other hand during chondrogenic differentiation of MSCs in periosteal of membranous bone in craniofacial skeleton such as avian quadratojugal joint (an equivalent to mammalian mandibular condylar cartilage), it was shown that N-CAM was not necessary before chondrogenesis (Fang J. *et al.* 1999). MSCs in gel-like biomaterials such as Col I, fibrin glue, Matrigel and PuraMatrix peptide hydrogel underwent proper chondrogeneis without a direct cell-cell communication (Dickhut A. *et al.* 2008) as well. Therefore, it seems that direct cell-cell interactions both *in vitro* and in *vivo,* are not always necessary for chondrogenic differentiation (Boeuf S. *et al.* 2010). However indirect paracrine communication between the cells in the gel-like material might play a role as it has shown that MSCs express different cytokine and growth factors (Liu C.H. *et al.* 2005; Kim D.H. *et al.* 2005).

In our study N-Cad remained down regulated in both groups of pellet and alginate bead until third week of differentiation and then it expressed in pellet and alginate culture on day 21. In limb bud experiment it was shown N-Cad expressed prior condrogensis in

condensed MSC and then it disappear from the centre of condensation while the cells continue their differentiation and then re-expressed in perichondrium, an indication of appositional growth (Oberlender S *et al.* 1994). As a comparison the expression of N-Cad in our experiment on day 21 can be justified with appositional growth of chondrogenic model of pellet in which the peripheral undifferentiated layer of cells behave as perichondrium (Hillel A.T. *et al.* 2010) or fibroblast (Alberts B. *et al.* 2002).

As limitation of this study is the limited time points up to three weeks, it has been reported that in 3-Dimentional woven scaffold hypertrophy depended to the duration of chondrogenesis (Dickhut A. *et al.* 2008) up to day 45, although in our study the gel like nature of alginate is different from the porous scaffold in the mentioned study, more investigation in longer time points in future studies is recommended.

4.6 Conclusion

Chondrogenic differentiated MSCs showed different characteristics in pellet and alginate cultures. Pellet culture showed a hypertrophic model, whilst in alginate there appears to be higher quality of chondrogenic differentiation process occurring with higher expression of chondrogenic genes and down-regulation of hypertrophic genes. The expression of adhesion molecules was not consistent with that of a normal chondrocytes. Since chondrocytes are solitary cells surrounded with ECM and are not in direct contact with each other, the expression of N-CAM1 and N-Cad may indicate the immature nature of chondrogneic differentiated MSCs in alginate culture. MSCs cultured in different experimental groups of alginate, pellet and monolayer, did not show the expression of chondrogenic or hypertrophic genes in control groups cultured in growth medium. These findings are consistent with morphological studies described in the previous chapter.

5 CHAPTER 5

Study 3: Treatment of full thickness articular cartilage defects in rabbit model with chondrogenic and undifferentiated bone marrow derived mesenchymal stromal cells loaded in alginate and alginate bead alone[1]

5.1 Study design

In this part of the study, rabbit mesenchymal stromal cells (MSCs) were isolated, expanded until passage 3 (P3) and characterized. The cells were transplanted into the rabbit (N=12) knee defects in two groups of chondrogenic group which were cultured in alginate beads for 21 days in chondrogenic medium, and non-chondrogenic group included undifferentiated MSCs (d0) loaded in alginate beads. In another group of rabbits (N=6), alginate beads transplanted without cells were performed in the knee defects. The rabbits were sacrificed after 3 and 6 months and the defects area of the knees were studied with gross morphological scoring (Brittberg scoring system), Histology (H&E, Safranin O Fast Green staining, O'Driscoll scoring), Immunohistochemistry for collagen type II, and biochemical analysis (GAG and protein).

[1] Parts of this project have been published at 1. J. Ortho. Res. 2011; 29(9):1336-42 2. 2. Am J Sports Med. 2012 Jan;40(1):83-90 and 3. Knee Surg Sports Traumatol Arthrosc. 2013 Oct 22.

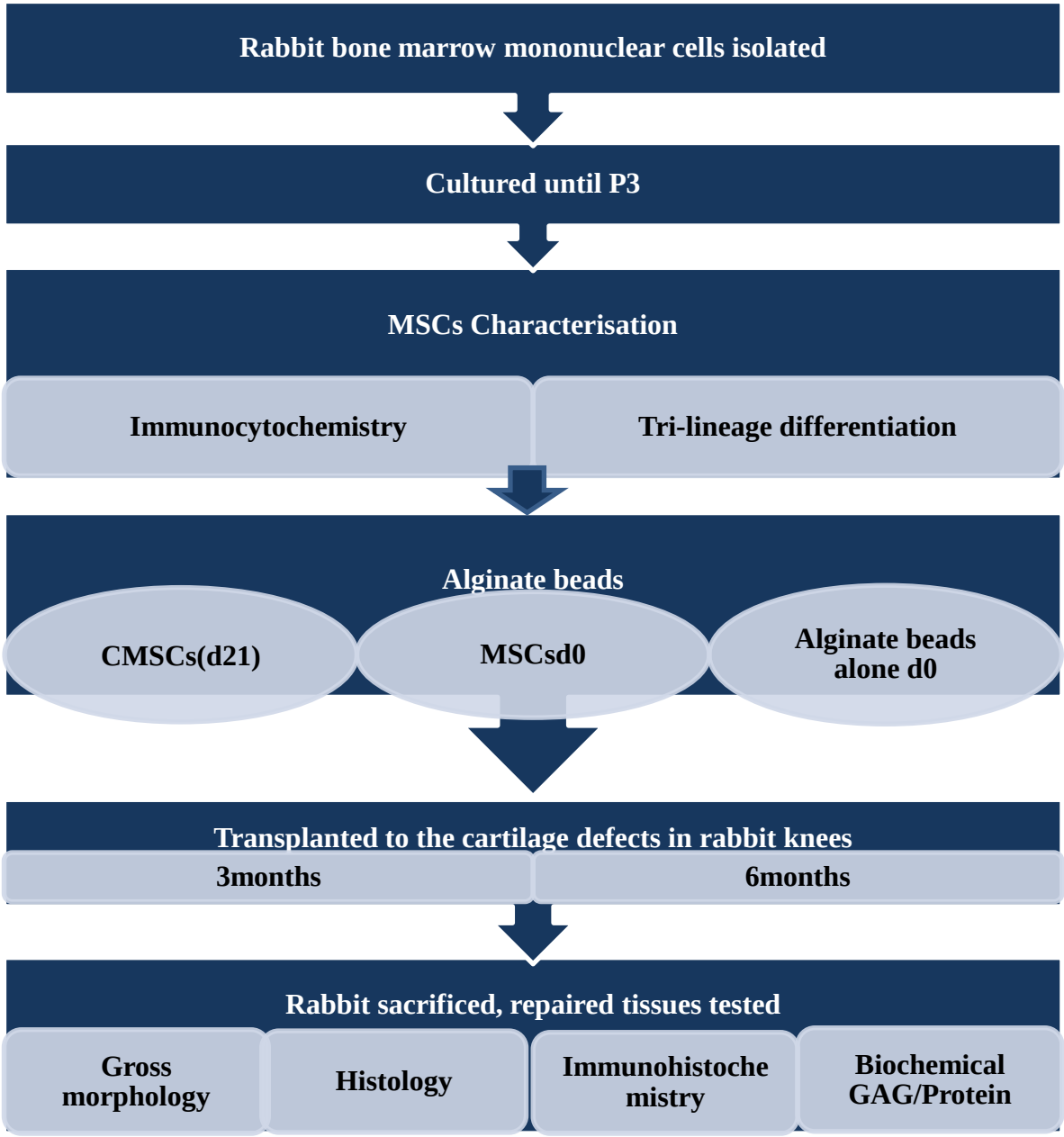


Figure 5-1 Flowchart shows the study design for chapter 5 *in vitro* and *in vivo* studies.

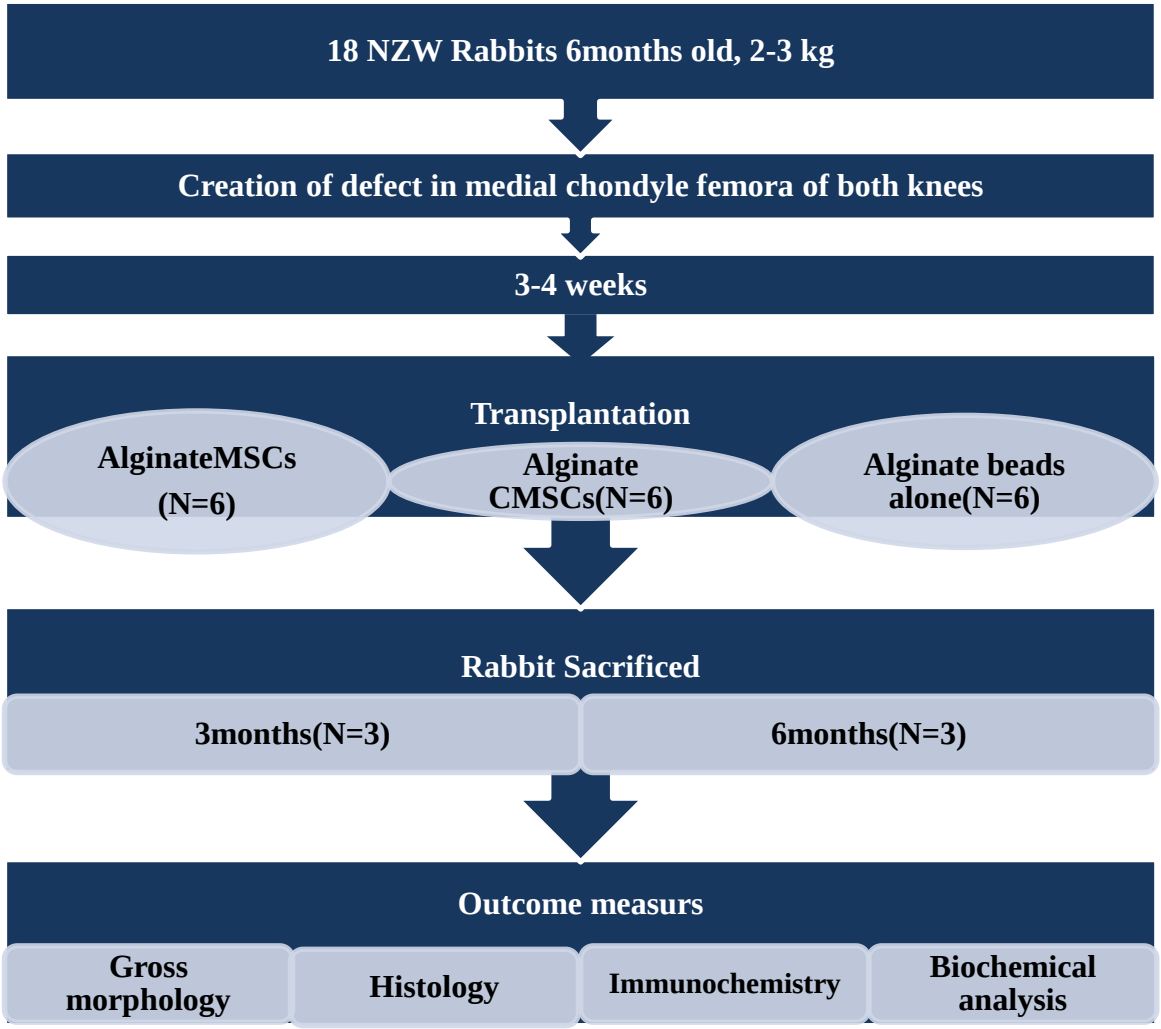


Figure 5-2 Flowchart shows the study design for chapter 5 *in vivo* study.

5.2 Introduction

Damage to cartilage tissue as the result of trauma or disease conditions are becoming increasingly common. The poor vascular properties of cartilage tissue make healing extremely difficult especially in the presence of full thickness articular cartilage defects. Cellular therapy has been considered a successful treatment option and has demonstrated to produce superior tissue repair quality as compared to conventional surgery in many studies (Wakitani S. *et al.* 1994; Brittberg M. *et al.* 1994; Fragonas E. *et al.* 2000; Wakitani S. *et al.* 2002; Brittberg M. 2010). Autologous chondrocyte implantation (ACI) has been used in many clinical trials and has been reported to produce good outcomes in previous literatures (Grande D.A. *et al.* 1989). However, the use of ACI has several limitations. These include donor site morbidity, cellular dedifferentiation when cultured *in vitro* and the reduced ability to maintain good long-term tissue repair (Temenoff J.S. *et al.* 2000). To overcome these issues, mesenchymal stem cells (MSCs) have emerged as an alternative cell source. One of the main characteristics of MSCs is its multipotency in which MSCs will continue to be a progenitor for other cell types despite being removed from its original environment (Pittenger M.F. *et al.* 1999). From previous studies, allogeneic or autologous bone marrow (BM) derived MSCs have been encapsulated into different scaffolds with or without the addition of growth factors to treat cartilage defects in various animal models. It has been reported that the transplantation of BM-derived MSCs in cartilage defects provide good repair outcomes (Colemana R.M *et al.* 2007, Guo X. *et al.* 2010). However, *in vivo* assessments of chondrogenic pre-differentiated MSCs (CSMC) in alginate to repair full thickness cartilage defect have not been previously demonstrated and therefore warrants further investigations. In previous chapters (3& 4), when chondrogenic induced MSCs using defined chondrogenic medium (hereto referred to as induced-MSCs) were compared to the MSCs that were cultured using growth medium

without the addition of any factors (hereto referred as non-induced MSCs), it became apparent that superior chondrogenesis using gene expression, biochemical, histological and immunohistochemical analysis, as well as ultra- structural studies can be observed. In this study, CMSC i.e. MSCs encapsulated in alginate gel cultured in chondrogenic medium, were implanted in cartilage defects and compared to MSCs loaded in alginate beads and transplanted without chondrogenic differentiation. The objective of this study was to compare the effectiveness of using non-induced MSCs and CMSC (or the terminologically similar induced-MSC) in alginate beads in order to evaluate the repair outcomes of full thickness cartilage defect in rabbit's knee at 3 and 6 months after injury. Moreover in order to investigate the role of alginate alone in the repaired tissue, in a separated group of rabbits (N=6) the defective knees were transplanted with alginate beads alone.

5.3 Methods and Materials

5.3.1 Experimental animals

Twenty New Zealand White male rabbits (N=20; 15- 16-week old, 2–3 kg) that were used in this study was in accordance with the guidelines of the Animal Care and Use Committee and, institutional review board in University of Malaya (Reference number: OS/10/11/2008/HD (R)). Two rabbits were sacrificed for bone marrow (BM) harvesting while the rest were used for the main experiment. BM was harvested in sterile conditions and the mononuclear layer (Fig. 5-1) was isolated as described earlier in chapter 2. The mononuclear cells was suspended in growth medium DMEM-LG supplemented with 10% foetal bovine serum (FBS) and cultured in 75cm^2 tissue culture flasks (Nunc, Rockside, Denmark). Cell culture medium was changed every 3 days and the cells were cultured and expanded until P3 in a humidified incubator at 37°C, 5%

CO_2. Cell viability was verified at each passage using TrypanBlue exclusion dye method prior to cell counting.

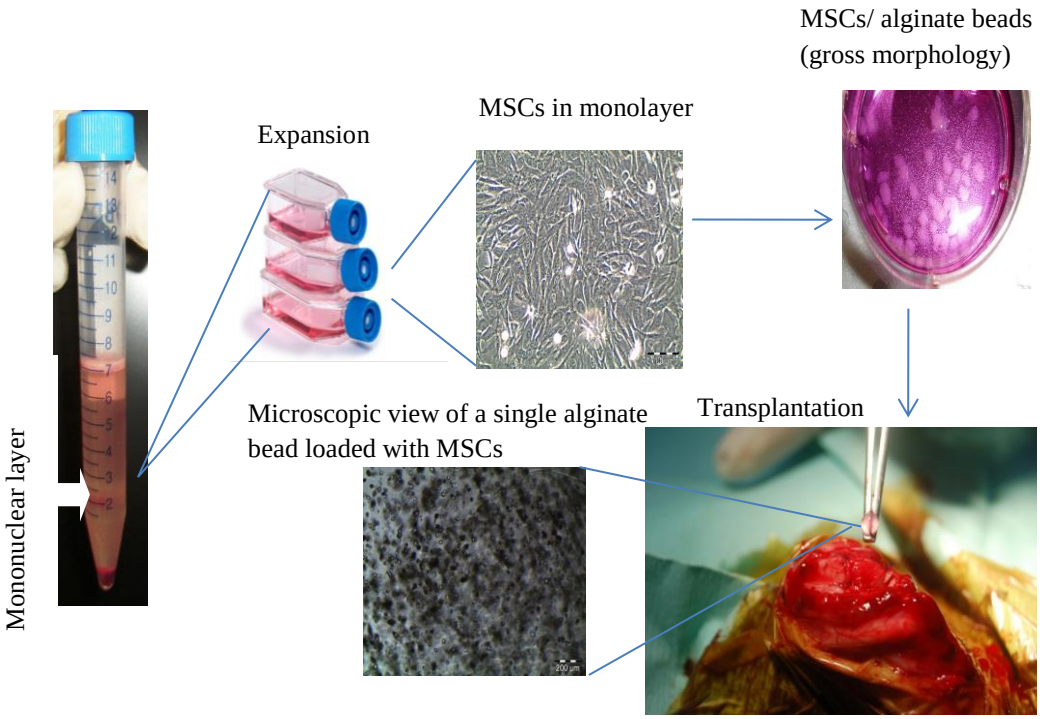


Figure 5-3 MSCs isolation, expansion, culture in alginate beads, and transplantation in rabbit knee

5.3.2 Preparation of alginate beads loaded with MSCs

MSC alginate constructs in growth medium were prepared just before being transplanted into the knee defects (Group 1; Fig. 5-1). Alginate bead loaded with cells, and cultured in chondrogenic medium (Group 2) as described earlier (3.3.2), Group3: alginate bead alone was dropped into $CaCl_2$ solution, and washed with PBS after 10 minutes and transferred to sterile medium for transplantation.

5.3.3 Creation of cartilage defects

Eighteen rabbits were equally divided into three groups. Each animal underwent surgery to create full thickness cartilage defects on the articular surface of the weight-bearing

medial femoral condyle of both knees (hind legs). The defects were created using a custom-made cylindrical chondrotome (Fig. 5-2), and standardized according to the following dimensions: 5 mm diameter and approximately 1 mm depth (or until cartilage tissue was completely removed, as observed under 5X magnification). Care was taken to not violate the subchondral bone. Total removal of cartilage tissue was confirmed using observations made under the aid of a microsurgical microscope.

5.3.4 Transplantation of alginate constructs and alginate beads alone

Transplantations of the MSCs (group 1) and CMSC (group 2) constructs or alginate bead alone (group 3) into the defective areas (i.e., **right knee** in each respective group) were conducted approximately 3–4 weeks following the initial defect creation procedure. The transplanted cells were obtained only from rabbits of the same litter. The **left knee** was left untreated as a control until the end of the experiment, representing the control group of the study. A periosteal flap was used to hold the alginate beads at the defect area using microsurgical sutures (Nylon 9-0) and fibrin glue (Fig. 5-2). Post-operatively, antibiotics and analgesia were administered for 3 consecutive days. No wound infection was observed in any of the rabbits. The rabbits moved freely within their cages and were sacrificed at 3 and 6 months after transplantation.

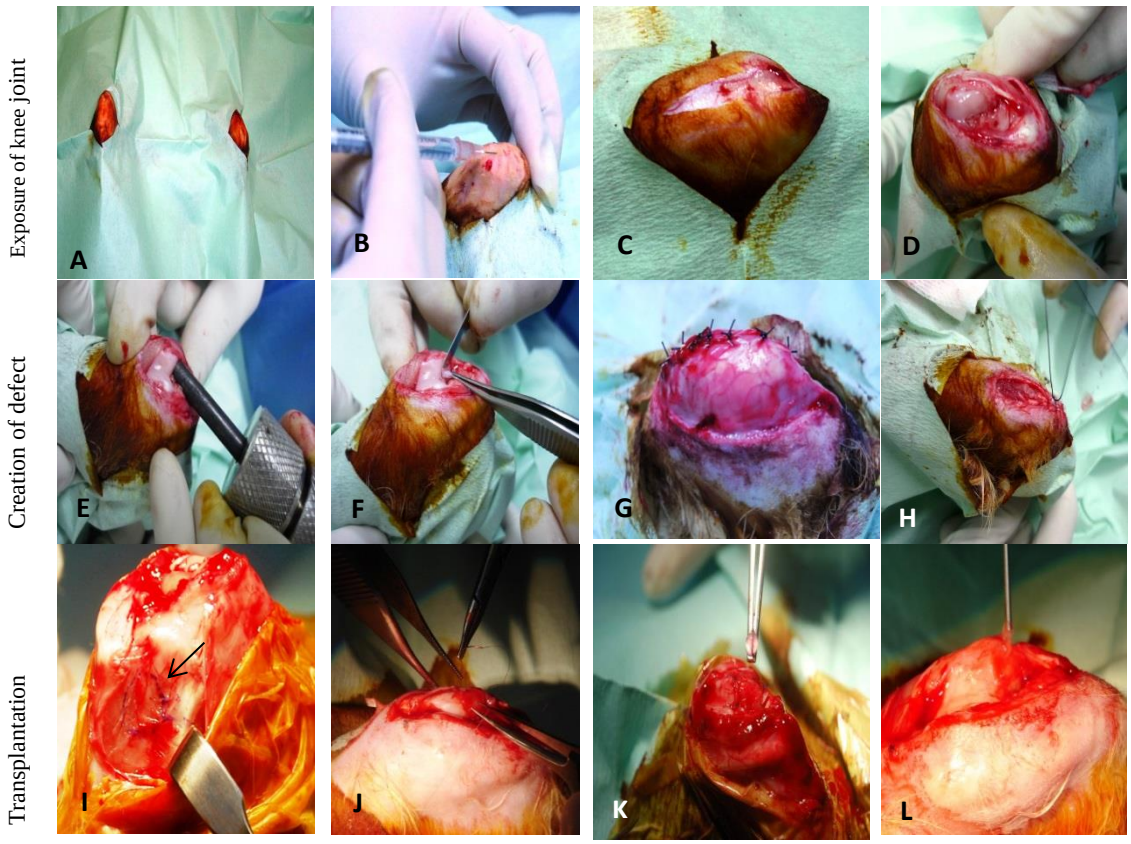


Figure 5-4 Surgical procedures

Upper panel: knee exposure

A. The knees were shaved and draped with sterile draping towel, and wiped with povidone- iodine.
B. Lignocaine 1% was injected subcutaneously as an anesthesia supplement.
C. Medial skin incision.
D. A parapatellar arthrotomy exposed the knee cartilage.

Middle panel: Creation of the defect in the femoral medial condyle of both knees.

E. Creation of the defect with a custom made chondrotome.
F. Removal of cartilage using a scalpel.
G. The soft tissue sutured back.
H. Skin was closed.

Lower panel: Transplantation 3-4 weeks after creation of defects in the right knee.

I. A periosteal flap was marked and separated from the upper medial side of tibia (arrow).
J. The periosteal flap with cambium layer faced down was sutured to the defect area of the right knee.
K. The constructs or alginate bead alone were placed under the periosteal flap.
L. The borders were sealed using fibrin glue.

5.3.5 Morphological Analysis

The quality of cartilage tissues in both knees were compared by two independent observers, in accordance to the Brittberg morphological score (Appendix G) (Brittberg M. 2000) and general histological evaluation.

5.3.6 Histology and Immunohistology

The distal femur was cut and the medial condyle was divided into two equal parts using a saw. Half of the samples used for histology. These samples were fixed in 10% buffered formalin for 48–72 hours, and decalcified in 10% formic acid. The specimens were processed for histological slides, embedded in paraffin, and cut into 5µm sections using a microtome. The slides were stained using haematoxylin and eosin (H&E), Safranin O Fast Green and immunohistochemical staining for collagen type II (Appendix B).

Immunohistochemistry for collagen type II was performed using primary antibody anti collagen type II (mouse-anti-rabbit; Merck, Darmstadt, Germany) in 1:100 dilution and according to the company protocol (DakoCytomation, Glostrup, Denmark). The histology images were analysed using O'Driscoll histological scoring as previously described (O'Driscoll S.W. 1988) (Appendix H).

5.3.7 Biochemical Analysis

Protein and glycosaminoglycan (GAG) were determined using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Richmond, CA) and Blyscan sulphated Glycosaminoglycan assay kit (Biocolor Ltd., Country Antrim, UK) according to the manufacturer's protocols. Spectrophotometer absorbance measurements were performed using a microplate reader (Epoch Bio-Tek) at 750 and 656nm for protein and

GAG assays, respectively. GAG content was normalized according to the protein contents (µg GAG/mg protein).

5.3.8 Statistical Analysis

Grading scores obtained from the Brittberg, O'Driscoll measures and GAG contents were statistically analysed using Wilcoxon signed-rank test available on the statistical software package SPSS (version 18). To compare the significant differences of values between the two experimental groups and, between treatment and control groups, Mann–Whitney tests were conducted accordingly. P value ≤ 0.05 was considered significant.

5.4 Results

5.4.1 Macroscopic Observation

The gross morphological appearances of the regenerated cartilage tissues filling in the defects at 3 and 6 months post-transplantation are shown (Fig.5- 3 B & D). The Brittberg's scores for the MSCs and CMSC groups were higher than their respective controls (8.17 ±1.83 and 7.00 ± 2.28 for MSC and CMSC, respectively). However these differences were not statistically significant (Fig. 5-7) at third month, but there were significant differences in the Brittberg score of transplanted knees in both groups treated with MSCs at 6 months (7.50 ±1.38 points) to that of the untreated knees (4.50 ± 1.38 points). CMSC-treated knees demonstrated better result as compared to MSC group (9.00 ± 2.00 vs. 7.33 ± 1.53). In group 3, i.e.: transplanted with alginate bead alone, the difference of Britterg's scores of right knees vs. left knees after 3 and 6 months (5.6±1.36 vs. 4.8 ± 1.437) was not statistically significant $p>0.05$ (Fig. 5-7).

Fig 5-3 D, illustrates the gross appearance of the regenerated tissue from CMSC treated and the untreated defect at 6 months post-transplantation. In the MSCs/CMSC treated knee, the regenerated cartilage tissues exhibited smooth glistening surface with less distinct demarcation of the defect border peripherally. In contrast, the regenerated tissues in the untreated knee and knees transplanted with alginate alone (Fig. 5-3 E & F) had rough surface and very distinct wound margins.

5.4.2 Sulphated Glycosaminoglycan (GAG) Analysis

The mean GAG/protein levels were higher in the treated knees, i.e. right knees, as compared to the control (left) knees at 3 and 6 months in MSCs group (Fig. 5-8). However, there was no significant difference between the mean GAG/protein levels of transplanted and control knee at 3 months in both groups of MSCs and CMSC. In comparison, the group of MSCs showed an increase in the GAG/protein content when compared to group CMSC at 3 months. At 6 months there was no significant differences between two groups of MSCs and CMSC, but the mean GAG level of the right knees were measured to be higher than the control, $p<0.05$ (Fig. 5-8). The GAG/protein content of group 3, showed no significant different in the right or left knees (0.968 ± 0.09 vs. 0.9 ± 0.08) $P>0.05$.

5.4.3 Histological and immunohistochemical analysis of specimens

The histology of the repaired tissue obtained from group MSCs and CMSC after 3 months showed a neocartilage contained immature tissue with clustered cells (Fig. 5-4 A, 5-4 E, 5-5 C&D, 5-6 A). The repaired tissue from CMSC at 6 months contained of both fibrocartilage and hyaline cartilage (Fig. 5-4 A). In the control left knees or alginate bead transplanted groups (both knees) the defect either were

unrepaired (Fig. 5-4 H, 5-5 G) or filled with fibrous tissue (Fig. 5-4 G), or incomplete repaired with a hyaline like cartilage, but it had not vertical integration with subchondral bone (Fig. 5-5 D) or included deep vertical fissures in the repaired tissue (Fig. 5-5 B, 5-6 G). This repaired tissue at 6 months was shown the primary sign of degeneration by deep surface fissures (Pearle A.D. *et al.* 2005; Koelling S. *et al.* 2009) in the neocartilage (Fig. 5-5 B). In some parts the defect was filled with bone (Fig. 5-5 B). Immunohsitochemical staining showed more concentration of type II collagen in transplanted tissues of both MSC and CMS (Fig. 5-6 A&C).

The GAG content of the repaired tissue was verified qualitatively by Safranin O Fast Green staining. In transplanted groups of MSCs and CMSC the repaired tissue stained with more intensity of maroon colour in comparison with that of the control in the intermediate and deep zones (Fig. 5-6).

O'Driscoll histological scores of the repair tissue was higher in the transplanted groups when compared to the untreated knees and alginate bead alone (10.5 ± 1.38 vs 7.67 ± 0.82 and 6.83±0.6) at 3 month, and (11.8 ± 1.47 vs. 8.67 ± 1.86 and 6.4±1.3) at 6 months post transplantation respectively, $p<0.05$. However, significant differences in scores were not apparent between the MSCs and CMSC group at 3 and 6 months (Fig. 5-9).

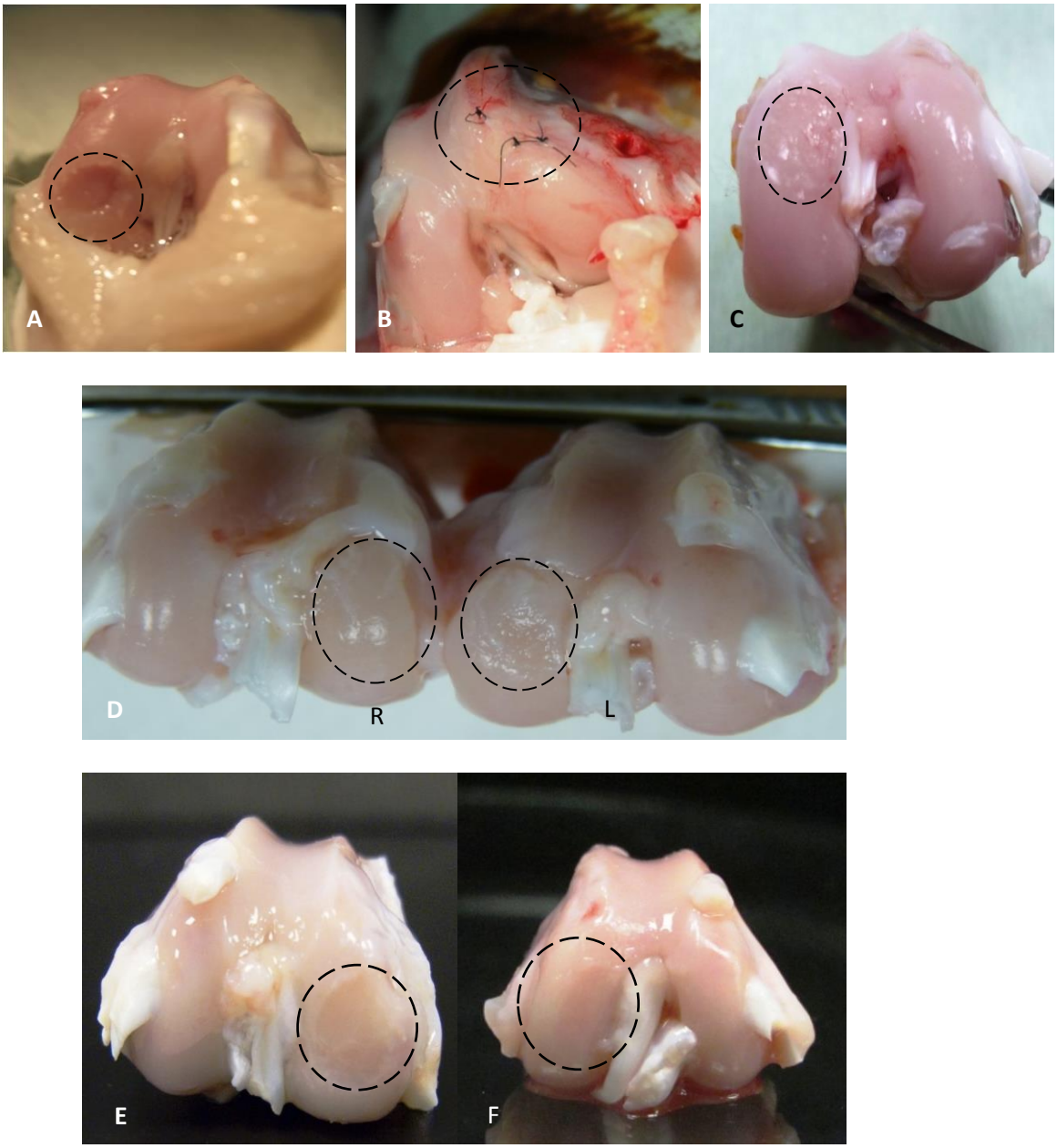


Figure 5-5 Gross morphology of rabbit knee in treated and control groups

A. Defect was created in the medial chondyle of the knee.
B. Right knee 3 months after MSC transplantation.
C. Left knee (control) defect with an irregular surface.
D. Right and Left knees of CMSC treated rabbits 6 months after transplantation. On the right knee repair tissue is more smooth and homogenous than the left knee (control) which was not treated.
E. Alginate beads alone transplanted at the right knee 6 months after transplantation.
F. Left knee control.

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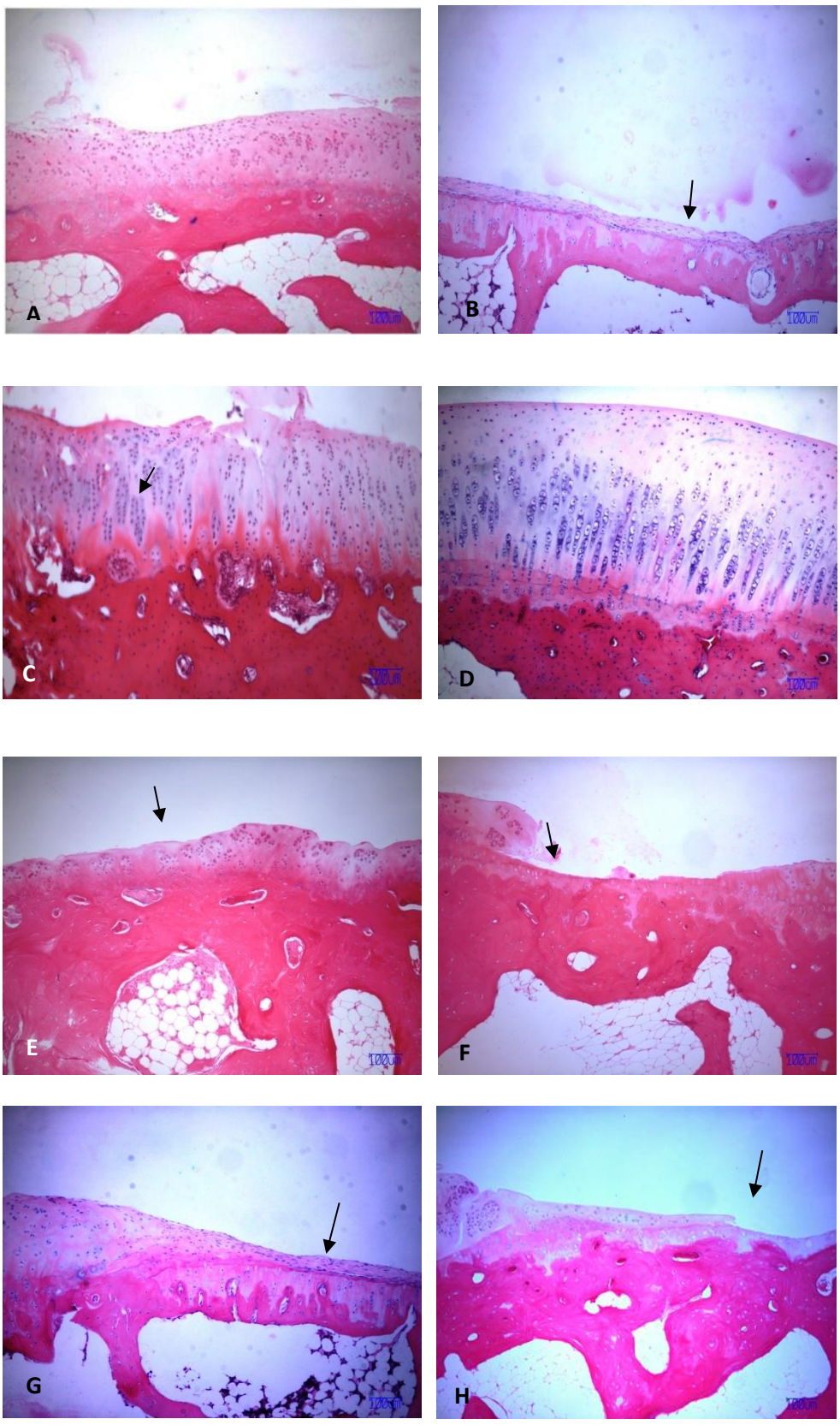

Figure 5-6 Histology of the defect site of the rabbit H& E staining

A. CMSC group 3months after transplantation, right knee
B. Left control arrow indicates fibrous tissue.
C. CMSC group right knee 6months after transplantation .Arrow shows the repaired tissue in the right knee composed of immature cartilage with clustered chondrocytes (arrow).
D. Normal articular cartilage of the rabbit knee.
E. MSC 3 months after transplantation in right knee. Arrow shows the repaired tissue.

F. Left knee control after 3 months.
G. Alginate alone transplanted group after 3 months. Arrow indicates fibrous tissue 10X.
H. Alginate alone transplanted group after 6 months. Arrow shows the fibrous tissue partially covered the defect site 10X.

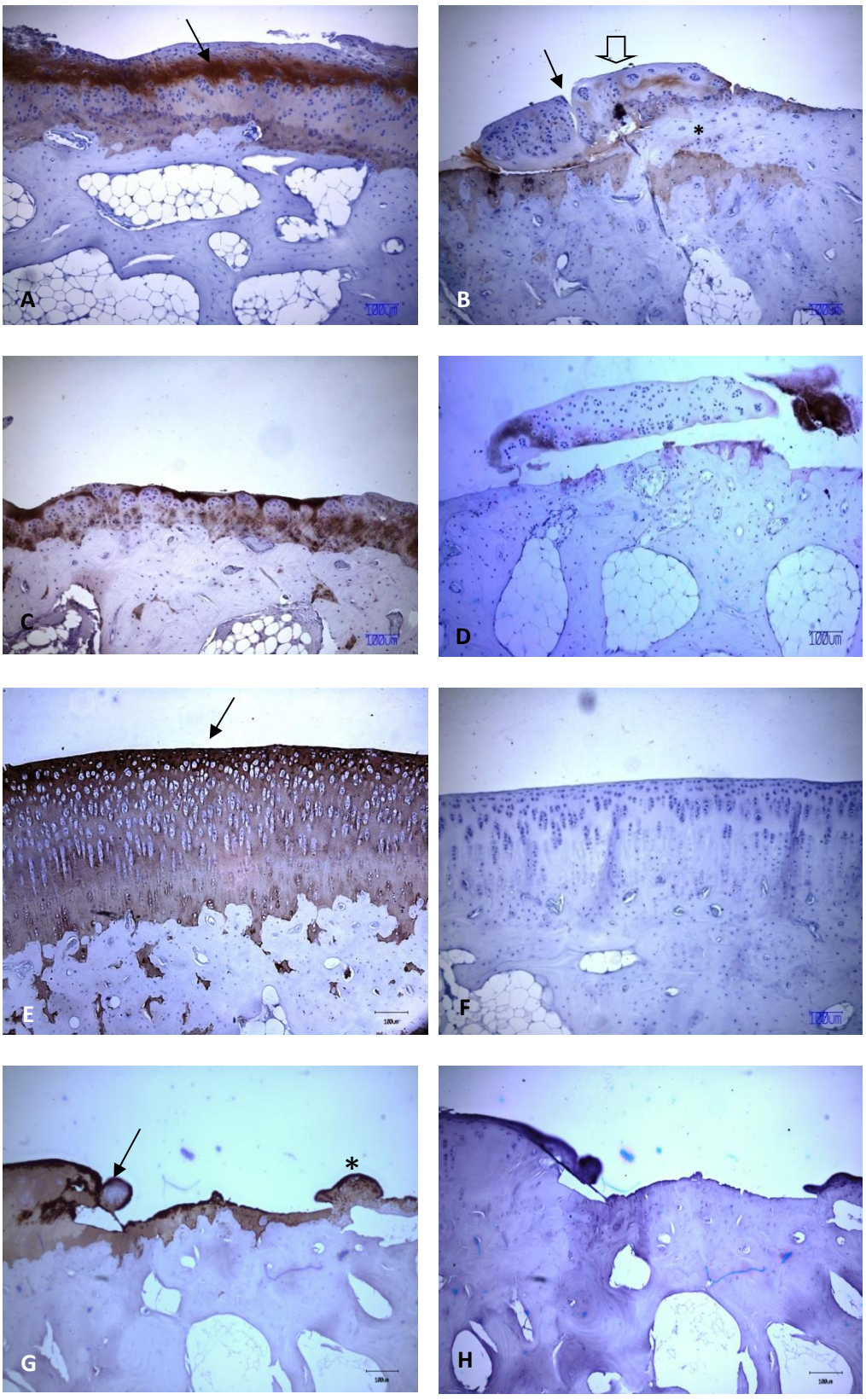

Figure 5-7 Immunohistochemistry for collagen type II in defect site of the rabbit knee

(A& B) CMSC 6months:

A. The repaired tissue of the right knee after 6 months contains a great amount of collagen type II especially at superficial layer (arrow).
B. Repaired tissue of the left knee shows an irregular immature cartilage mass with clustered cells and low amount of collagen type II (open arrow), a part of the defect has been replaced by bone (astriks) , vertical fisure at the repaired cartilage (thick arrow).

(C&D) MSC 3months treated group:

C. Collagen type II is deposited (stained brown) in the repaired cartilage in MSC treated site after 3 months.
D. The defect of the left knee after 3 months, the repaired cartilage has no vertical integration with subchondral bone, it contains scarce ammount of collagen type II (arrow).

(E & F) normal cartilage:

E. Normal cartilage as positive control type II collagen is more aboundant in superficial zone (arrow).
F. Normal cartilage negative control (stained without collagen type II antibody).

(G& H) alginate alone:

G. Alginate bead alone transplanted knee after 6 months, stained for collagen type II with immunohistochemistry; arrow indicates the edge of the defect. The brown color indicates collagen type II, at the defect area it shows the remaining of calcified layer. A small inhomogeneous patch of neocartilage is seen at the defect site, astriks 10X.
H. Negative control 10X.

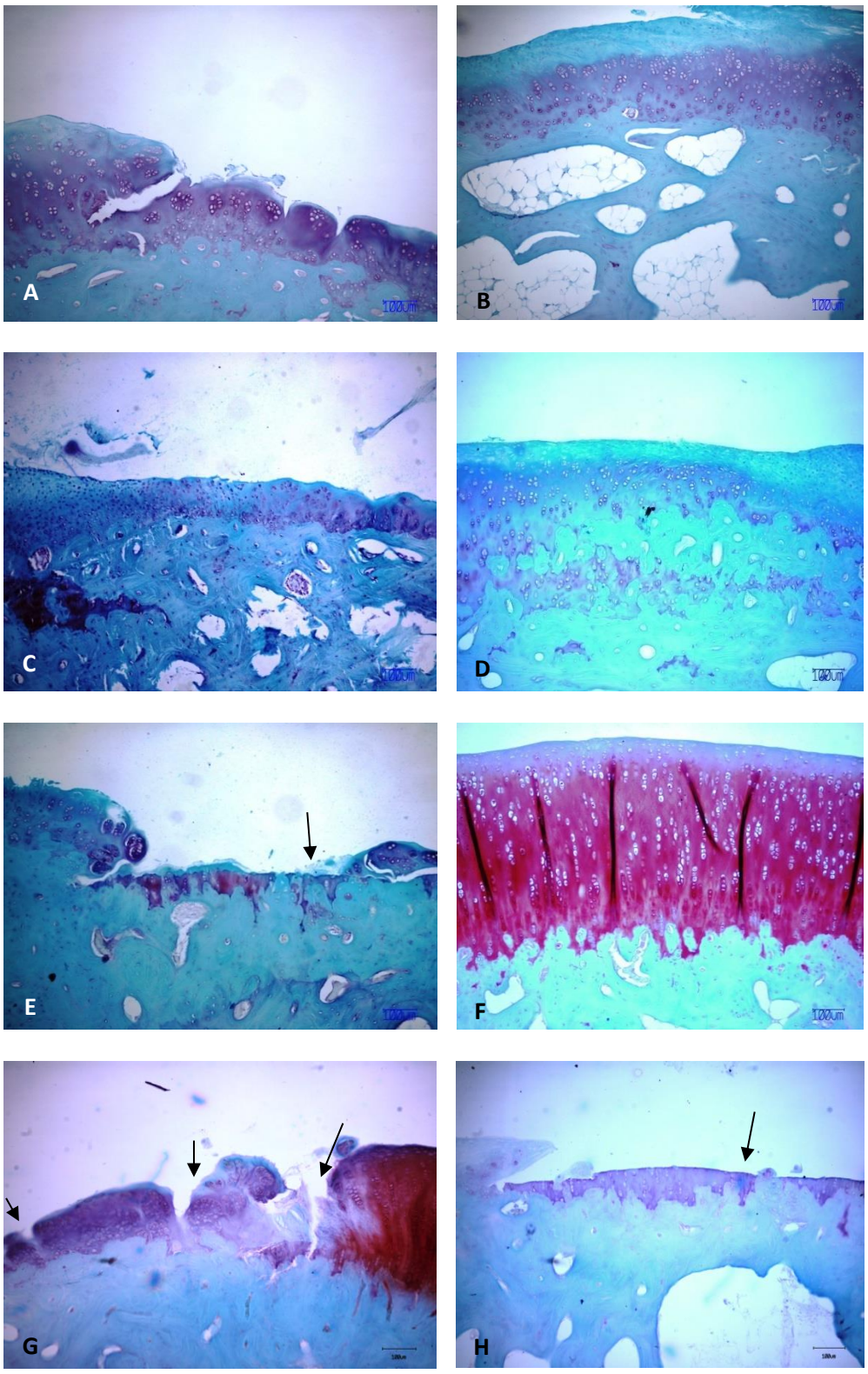

Figure 5-8 Safranin O Fast Green staining of the repaired tissue in rabbit knee

(A&B) CMSC group:

A. 3 months after transplantation.
B. 6 months after transplantation.

(C & D) MSCs group right knee:

C. 3 months after transplantation.
D. 6 months after transplantation.
E. Left control, the defect site shows no or scarce amount of repaired tissue (arrow).

F. Normal cartilage, intermediate and deep layers stains purple an indication of higher glycosaminoglycan, superficial layer and bone stains green.
G. Right knee 6 months after transplantation of alginate bead alone, the right side of the picture shows native cartilage (maroon colour), and the repaired tissue is discontinues with vertical fissures (arrow).
H. Left knee control shows no healing at the defect site (arrow).

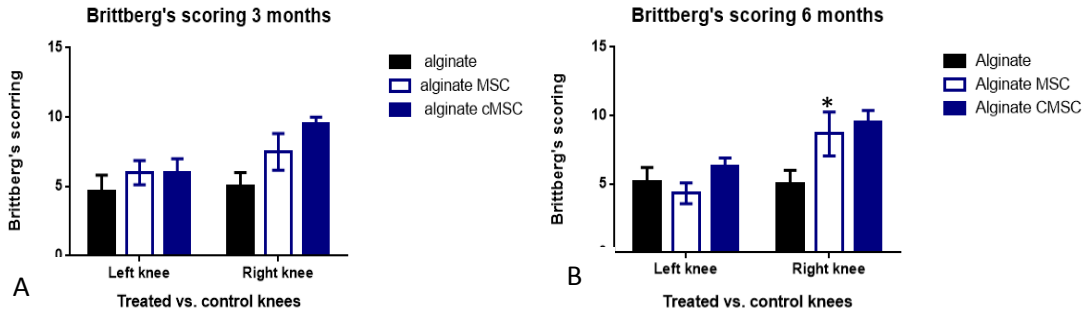

Figure 5-9 Brittberg's scoring

Brittberg score comparing MSCs and CMSC treated knees (Right) to non-treated knees (Left) in (A) 3 and (B) 6 months *p≤0.05.

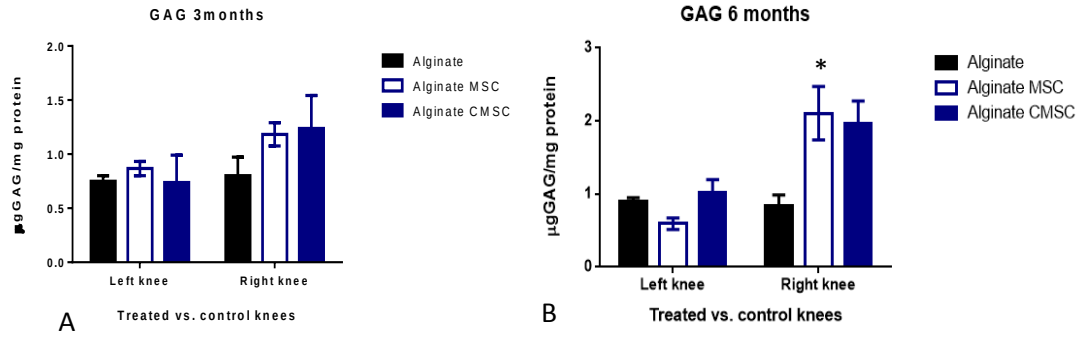

Figure 5-10 Biochemical analysis of sulphated GAGs content in experimental groups of rabbit knee

GAG/total protein levels comparing MSC and CMSC treated knees (Right) to non-treated knees (Left) at (A) 3 and (B) 6 months (error bars= SD) *p≤0.05.

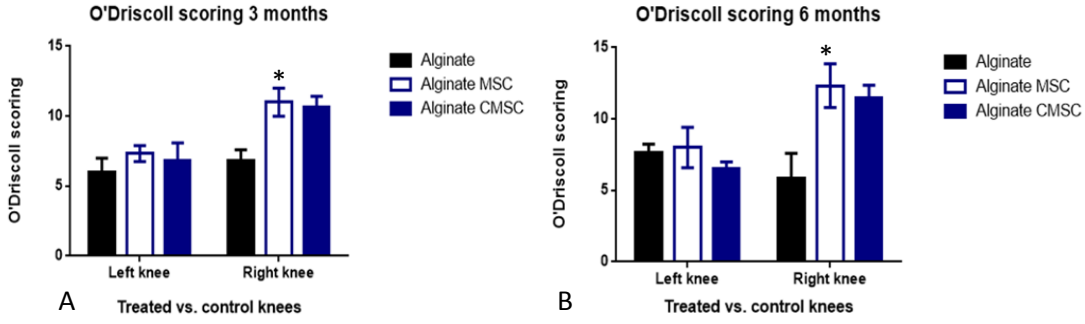

Figure 5-11 Histological scoring (O'Driscoll scoring)

O'Driscoll score comparing MSCs and CMSC treated knees to non-treated knees (left) (A) 3 and (B) 6 months (Error bars=SD) *p≤0.05.

5.5 Discussion

Mesenchymal stem cells exhibit a stable phenotype *in vitro* and have multilineage potential of differentiating into the adipocytes, chondrocytes, or osteocytes (Pittenger M.F. *et al.* 1999). *In vitro* studies have suggested that the induction of adult stem cells into chondrogenic lineage could be achieved via the introduction of soluble, biophysical, and 3D culturing environment factors (Guilak F. *et al.* 2009). In this study both cell types i.e. MSCs vs. CMSC were encapsulated in alginate gel, the 3D environment provided by the alginate gel may have maintained the spherical cell shape, thereby promoting chonrogenesis. It has been shown that the differentiation of MSCs into a chondrogenic phenotype requires the cells to maintain a rounded shape (Guilak F. *et al.* 2009), which can be achieved through encapsulation within a semi-solid material, such as that provided by the alginate gel. Moreover compression shear loading in a bioreactor was shown to induce MSCs seeded in a Fibrin-polyurethane composite, production of endogenous TGFβ3 and subsequently upregulation of the cartilage specific genes including Collagen type II (Li Z. *et al.* 2010; Schätti O. *et al.* 2011). Henrionnet C. *et al.* (2012) reported expression of chondrogenic markers under mechanical agitation in human bone MSCs cultured on alginate beads without TGF-β treatment. There is a possibility that MSCs loaded in alginate bead after transplantation in the knee defect site sustained the same mechanical loading and as a consequence differentiated to chondrocyte without being induced to chondrogenic differentiation in advance. Therefore after 6 months there was no significant difference between GAG/protein quantity of the repaired tissue in both MSC and CMSC treated knees, yet significantly higher than empty defects and defects transplanted with alginate beads alone.

In the present study, the gross morphological appearances of smoother and more homogenous regenerated cartilage tissues in the MSCs-treated knee indicated a superior tissue repair following MSCs treatment compared those left untreated. This finding

was consistent with previous reports demonstrating satisfactory repair of damaged cartilage with the use of MSCs, even in conditions involving osteochondral defects (Wakitani S. *et al.* 1994; Wakitani S. *et al.* 2002). The question however remains as to whether the observed enhanced cartilage repair was a direct result of the transplanted MSCs or merely a consequence of augmented expansion of the surrounding cells, for example, chondrocytes/progenitor cells or extracellular matrix, contributing to the healing process (pelttari K. *et al.* 2008). To the best of our knowledge, this issue has not been specifically addressed in any previous publications. However the possibility of eliciting cartilage repair using alginate itself was ruled out as in alginate bead alone transplanted in the defect knees there was no superior repaired tissue when it compared with controls in 3 and 6 months after transplantation .This result is similar to (Fragonas E. *et al.* 2000) study in which the defect filled with alginate alone were produced a fibrous tissue, similar to the defect without any treatment.

The histological analyses of the present study identified clear differences between the MSCs-treated and untreated knees. The MSCs treatment appeared to induce a greater amount of type II collagen formation. In contrast, the repaired tissue of the untreated knee showed an irregular immature cartilage mass with clustered cells and low amount of collagen type II. In addition, the repaired tissue treated with MSCs contained a vast amount of GAG in the intermediate and deep zones, whereas the untreated regenerated tissue showed only scarce amount of GAG. Similar observation was reported in a previous study (Diduch D.R. *et al.* 2000), demonstrating that cartilage treated with MSCs had extracellular matrix that primarily consisted of a hyaline and fibro-cartilage mixture, while the untreated cartilage defects were composed of merely a thin layer of

fibrous tissue. There are a number of limitations in the study that are worth highlighting. The present study utilized only a limited numbers of animals as a consequence of financial constraints. This prohibits the use of parametric statistical analyses, which would in effect, produce more convincing results, and thereby strengthen the conclusions derived from the analyses. In addition, the constraints related to the availability of resources allowed assessment of cartilage repair only at 2 time points, that is, 3 months and 6 months post-surgery. A larger number of observational time points that should ideally be performed would provide further information regarding the temporal evolution of the repair process, providing a better insight into the actual effect of MSCs on the long-term outcome of cartilage repair.

It was not possible to identify the percentage of MSCs that underwent chondrogenic transformation due to the technical complexities involved. In addition this meant that the homogeneity of the CMSC used in the present study was also not verified. However, the characterization of CMSC has provided strong evidences to support that chondrogenic transformation *in vitro* has occurred to a large degree as demonstrated by the strong positive staining of GAG and collagen type II. Lastly, the present study only investigated the anatomical profiles of the regenerated cartilage as well as its extracellular matrix biochemical profiles. The introduction of more sophisticated investigative modalities such as proteomic and gene expression analyses would provide more precise molecular markers of the regenerate cartilage tissue, thus providing a global depiction of the healing process with and without MSCs treatment.

5.6 Conclusion

In conclusion, this study demonstrates that the transplantation of MSCs loaded in alginate as a carrier for repair of full thickness articular cartilage defects produced

superior healing compared to the intrinsic repair of the untreated cartilage defects, irrespective of their state of differentiation. This has a clinical implication on wider use of the undifferentiated MSCs for cell-based transplantation therapy for articular cartilage repair, as this option is associated with more simplified laboratory processing that would not impose on additional resources and financial burden. It also proved the safety and effectiveness of alginate as a cell carrier for cartilage damage treatment.

6 CHAPTER 6

General discussion and future work

Alginate is an inert biomaterial with poor cell attachment quality. Although this trait can be a disadvantage for the differentiation of some adherent dependent cells such as osteoblast during osteogenic differentiation (Alsberg E. *et al.* 2001), its benefit for chondrogenic differentiation of MSCs appears to be quite relevant. It is apparent that MSCs loaded in alginate system maintain a spherical phenotype that is a pre-requisite for chondrogenic differentiation to occur. Moreover the cells surrounded with a hydrated microenvironment provide a similar cellular milieu to that of articular cartilage ECM. When stimulated with chondrogenic inducing factors such as TGFβ3, cells produce a high amount of cartilage matrix including collagen type II and interact with through integrin molecules. These events consequently are involved in complex signalling pathways which include establishing cell polarity and maintaining cell growth and survival (Ramage L. *et al.* 2012).

Cell proliferation studies of chondrogenic groups showed cell number in alginate significantly increased over time, while pellet culture remained unchanged and in monolayer decreases significantly. These differences in cell proliferation in different systems can be explained with hypertrophy in pellet and monolayer, as it was shown by higher expression of hypertrophic genes in these groups. It has been shown that hypertrophic chondrocytes withdraw from the cell cycle and stop proliferation after certain period of time (Hunziker E.B. *et al.* 1987).

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In this study MSCs loaded in alginate beads and cultured in chondrogenic medium, as determined in previous studies, showed a non-hypertrophic articular cartilage-like morphology. This finding is contentious since there are those who have similar findings (Yang I.H. *et al.* 2004, Andriamanalijaona R. *et al.* 2008) whilst others, whom reported the expression of hypertrophic factors during chondrogenic differentiation of MSCs in alginate beads, would disagree (Steinert A. *et al.* 2003, Ichinose S. *et al.* 2005, Xu J. *et al.* 2008, Bian L. *et al.* 2011, Fernandes A.M., *et al.*2013). Further studies would therefore be required to further elucidate the process with more encompassing analyses of the events occurring to elucidate the reasons for these varying observations. Nevertheless, this study at present does provide a valuable opportunity for us and many others to explore more about the underlying factors that resulted in different outcomes in each of these experiment; i.e. to optimize a truly non-hypertrophic model for cartilage tissue engineering application.

To further investigate the underlying mechanisms of the different chondrogenic differentiation systems, i.e.: alginate bead, pellet culture and monolayer, a study of TGFβ receptors activity during such chondrogenic differentiation of MSCs is recommended. A recent study using ectopic expression of TGFβ receptor type II (TβRII) has shown that this receptor has an important role in the maintenance of the chondrocyte phenotype during successive passages in monolayer (Baugé C. *et al*.2013).

It is worth noting that the expression of collagen type I and adhesion molecules reduces the optimum quality of the engineered cartilage. A low oxygen tension environment is recommended to test chondrogenic differentiation of MSCs cultured in alginate. It has been shown that the expression of Col I was reduced (Meyer E.G. *et al.* 2010; Sheehy E.J. *et al.* 2012) during chondrogneic differentiation of MSCs in low oxygen culture environment. Chondrogenic differentiation of MSCs under a low oxygen tension can be further investigated for the abundance and morphology of mitochondria following

chondrogenic differentiation. It is our suggestion that the use of immunofluorescent and transmission electron microscopies be employed to demonstrate these and other changes.

Our *in vivo* study showed that a full thickness cartilage defect which had not penetrated to the subchondral bone may benefit for the transplant for cells or cell-scaffold construct. The use of alginate with cells (undifferentiated or chondrogenic differentiated) resulted in a superior repair outcome compared with non-treated knee cartilage defects. The important findings in this thesis were that transplantation of alginate alone had no modulatory effect on cartilage repair. This suggests that for cartilage repair to take place, cells play a vital role in the remodelling process and thus is the main factor that provides superior cartilage repair outcomes in cell therapy. However, the issue as to whether it is the cells themselves, the factors they secrete or the immunomodulatory effect that results in this positive outcome remains a hotly debated issue with no clear consensus at the moment (Caplan A. I. *et al*. 2006; Iyer S.S. *et al.* 2008, Anderson J.A. *et al.* 2013). Our study lacks this component that explains the positive outcome observed as it was not part of the original study design nor of the main research question. If we were to attribute the outcome to the cells themselves, cell tracking experiment could be employed. Incorporating this in future studies to address the cell fate following transplantation of cell alginate constructs between the two treatment groups would be a prudent move and may provide the answers for these questions. Improvement could also be made to the *in vivo* study by using autologous MSCs instead of allogeneic sources, as it has been shown that *in vitro* or *in vivo* chondrogenic differentiation of MSCs with or without an alginate matrix increased immunogenicity and anti-donor immune response in MSCs (Ryan A.E. *et al.* 2014). This, however, can only be performed in larger animal models, given that extracting

MSCs from rabbit marrow may prove to be too challenging. In addition, higher number of animals will allow using parametric test with a higher degree of reliability obtained.

Collagen based cell free scaffold combined with microfracture showed good results of cartilage repair in clinical studies (Freymann U. *et al.* 2013), but in our experiment alginate alone did not elicit any chondral repair. This can be due to firstly: the defect was not penetrated to the subchondral bone therefore MSCs had no access from bone marrow to the repair site. Secondly, alginate is a negatively charged gel that does not attract any cells to be incorporated into the repair tissue. Perhaps an active cell free alginate scaffold, a modified shape of alginate covalently bonded with adhesive peptides such as RGD in an osteochondral defect, or a chondral defect coupled with microfractue, would have more satisfactory results.

Although the repair tissue resulted from transplantation of chondrogenic alginate group in rabbit was similar to the outcome of non-induced group, it would not decrease the importance of chondrogenic differentiation studies of MSCs in alginate beads *in vitro.* In another study using direct parallel comparison between allogeneic MSCs and autologous chondrocyte for repair of focal cartilage defects of rabbit knee, we observed similar outcome of morphology and biochemical profiles (Tay L.X. *et al*, 2012). The results indicate the feasibility of using undifferentiated MSCs loaded in alginate for treatment of cartilage injuries, while a chondrogenic differentiated model in alginate beads can be a suitable *in vitro* model for studying the underlying mechanism of hypertrophy during *in vitro* chondrogenic differentiation.

In addition to the limitations that mentioned earlier, there are several others worth mentioning in order to not overstate the finding of the present thesis. This study would be more convincing if proteins were studied using immunochemistry, western blotting, or protein profile of the constructs. This is especially true for hypertrophic markers such

as collagen type X and RunX2 and adhesion molecules of NCAM1 and N-Cadherin. In the in *vivo* study, although Safranin O Fast Green specifically stained proteoglycans also showed the non-chondrogenic area of the repaired tissue, most probably contained collagen type I. However, Col I could have been studied in the repaired tissue using more specific staining such as immunohistochemistry. There were limitations with time and finance that prevented us for further studies such as protein expression. Therefore more protein expression studies are recommended, for which it is hoped that such undertaking may be conducted in the near future.

7 CHAPTER 7

Conclusions

To summarize the conclusions of the *in vitro* and *in vivo* investigations using alginate as a scaffold or cell carrier for cartilage repair as described in the present thesis:

1. Chondrogenic differentiated MSCs in alginate beads were more similar to cells in a healthy articular cartilage like model, than that of a hypertrophic model.
2. MSCs in pellet culture and monolayer culture represented a hypertrophic model of chondrogenesis when subjected to chondrogenic differentiation processes.
3. Pellet cultures resulted in significant cell death and therefore the use of scaffolds/carriers like alginate is preferred since it allows cells to be viable whilst supporting chondrogenesis.
4. Expression of adhesion molecules N-CAM1 and N-Cad may indicate the immature nature of chondrogneic differentiated MSCs in alginate hydrogels.
5. Superior chondrogenic expression is observed in alginate culture and therefore is preferable to pellet culture.
6. Transplantation of MSCs in cartilage defects showed similar results as transplantation of CMSC.
7. Cell loaded alginate produced superior repair tissue, especially 6 months after the transplantation in the cartilage defects.
8. Alginate bead alone did not support or induce any repair tissue after being transplanted in the defected knee cartilage.
9. In the overall, alginate beads offer great potential as a cell carrier that provides the ideal environment for chondrogenic differentiation of MSCs *in vitro* and *in vivo,* and with further improvement on the chondrogenic conditions, may prove

itself as the most potential candidate as a promising scaffold for clinical application to be used for repairing damaged cartilage.

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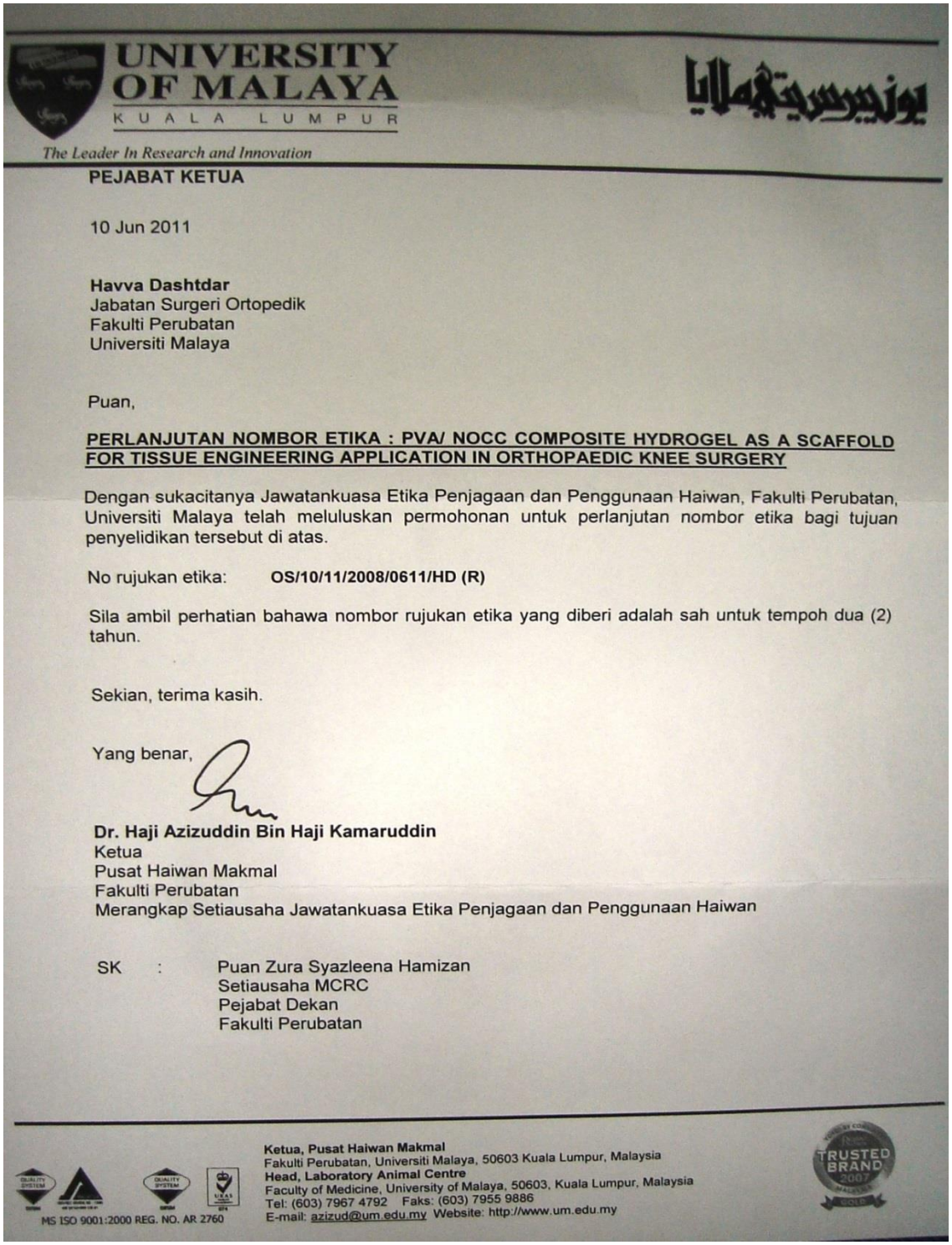
APPENDICES

APPENDIX A: (a) Approval letter from university of Malaya Animal Care and Use



UNIVERSITY OF MALAYA
KUALA LUMPUR

يونيورسيتي مالايا

The Leader In Research and Innovation

PEJABAT KETUA

10 Jun 2011

Havva Dashtdar
Jabatan Surgeri Ortopedik
Fakulti Perubatan
Universiti Malaya

Puan,

PERLANJUTAN NOMBOR ETIKA : PVA/ NOCC COMPOSITE HYDROGEL AS A SCAFFOLD FOR TISSUE ENGINEERING APPLICATION IN ORTHOPAEDIC KNEE SURGERY

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk perlanjutan nombor etika bagi tujuan penyelidikan tersebut di atas.

No rujukan etika: **OS/10/11/2008/0611/HD (R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh dua (2) tahun.

Sekian, terima kasih.

Yang benar,

Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : Puan Zura Syazleena Hamizan
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

MS ISO 9001:2000 REG. NO. AR 2760

Ketua, Pusat Haiwan Makmal
Fakulti Perubatan, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
Head, Laboratory Animal Centre
Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia
Tel: (603) 7967 4792 Faks: (603) 7955 9886
E-mail: azizud@um.edu.my Website: http://www.um.edu.my

TRUSTED BRAND 2007

Translation of the letter

10 June 2011

Havva Dashtdar
Department of Orthopaedic Surgery
Faculty of Medicine
University of Malaya

MS,

Extension of Ethics Number: PVA/NOCC Compostite Hydrogel as a Scaffold for Tissue Engineering Application in Orthopaedic Knee Surgery

With regards to above matter Animal Care and Use Ethics Committee, Faculty of Medicine, University of Malaya has extended the application for the above research.

Ethics approval reference number: **OS/10/11/2008/0611/HD(R)**

Please be advised that the above ethics approval reference number is valid for two years.

Thank you

Yours Sincerely

Dr. Haji Azizuddin Bin Haji Kamaruddin
Head
Animal Research Centre
Faculty of Medicine
Secretary of the Ethics Committee of Animal Care and Use

CC: MS Zura Syazleena Hamizan
Secretary MCRC
Dean office
Faculty of Medicine

APPENDIX: A (b) Approval letter from Medical Ethics Committee, University Malaya Medical Centre

PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79564422, 03-79574422 KEBEL: UNIHOS, KUALA LUMPUR
FAX NO: 6-03-79545682

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre **ADDRESS:** LEMBAH PANTAI 591.00 KUALA LUMPUR	**ETHICS COMMITTEE/IRB REFERENCE NUMBER:** 472.95
PROTOCOL NO: A5951002 **TITLE:** The Effects Of Different Factors On Differentiation Of Human Mesenchymal Stem Cell Into Chondrocytes.	
PRINCIPAL INVESTIGATOR: Dr. Tunku Kamarul Zaman **TELEPHONE:** **KOMTEL:**	**SPONSOR:**

The following item [✓] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[ ] Borang Permohonan Penyelidikan	Ver date:
[ ] Study Protocol	Ver date:
[ ] Investigator's Brochure	Ver date:
[✓] Co-Investigator (Ms. Chong Pan Pan) replace Nurasikin Salim	Ver date:
[ ] Consent Form	Ver date:
[ ] Advertisement/Payment & Compensation to Subjects	Ver date:
[ ] Investigator(s) CV's (if applicable)	

and have been [✓]

[✓] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Comments:

Date of approval: 16th November 2005

PROF. LOOI LAI MENG
Chairman
Medical Ethics Committee

APPENDIX B: Histological staining protocol

A. Hematoxylin and Eosin staining (H&E)

In order to study the general morphology of tissues, cells, or constructs H&E staining was performed according to the following protocol:

Reagents:

1. Hematoxylin and Eosin (Sigma-Aldrich) were filtered before use.

2. Acid alcohol 0.25ml of HCl in 100ml of 70% alcohol

3. Bluing reagent: 0.037 mol/L of Amonium persulphate in distilled water

Protocol:

1. Deparafinized the paraffin sections in 3 changes of Xylene each 2 minutes.
2. Rehydrate the sections through ascending series of ethanol (100, 95, 70 each 2minutes, water up to 5minutes).
3. Stain in Harris Hematoxylin 2 minutes.
4. Rinse slides in running tap water.
5. Plunge in acid alcohol 1-2 dips as a differentiation reagent.
6. Rinse with tap water.
7. Dip in bluing reagent for 5-60 seconds.
8. Rinse in water or alcohol (if using the aqueous based Eosin, rinse the slides with water. For alcoholic based Eosin rinse slides in 95% ethanol).
9. Place the slides in Eosin for 30-60 seconds.
10. Wash in distilled water.
11. Dehydrate in an ascending series of ethanol (70, 95, and 100 each 2 minutes).
12. Clear in two changes of Xylene each 2 minutes.
13. Mount with DPX and Cover Slides.

B. Safranin O staining for micromass culture:

1. Fix the cells with 10% formalin for 20 minutes.
2. Rinse with PBS 3 times.
3. Rinse with 1% acetic acid 10 seconds.
4. Stain in 0.1% Safranin O solution for 5 minutes.
5. Rinse twice with PBS.
6. Keep in ddH2O or PBS (or glycerin).
7. Record pictures.

C. AlcianBlue staining for micromass culture:

Reagents: AlcianBlue (Sigma-Aldrich) 1g per 100ml of 3% acetic acid PH=2.5

1. Fix the cells with 10% formalin for 20minutes.
2. Rinse the cells 3 times with PBS.
3. Stain with AlcianBlue for 1-2hrs.
4. Rinse with 3% acetic acid.
5. Rinse with PBS twice.
6. Keep in ddH_2O or PBS (or glycerin).

D. Safranin O Fast green staining for tissue paraffin sections.

Reagents:

1. 1.5% aqueous safranin O in distilled water.
2. 0.02% alcohol Fast green: (0.02 g fast green in 100ml 95% ethanol).
3. 1% acetic acid (always use fresh).

Procedure:

1. Deparafinized sections in 3 changes of Xylene 5 minutes each.
2. Rinse in 2 changes of 100% ethanol 1minute each to remove Xylene.
3. Hydrate sections in 2 changes of 95% ethanol 1minute each.

4. Finish hydration with 3 changes of distilled water, 1 minute each.
5. Put slides in 1. 5% Safranin O for 40 minutes.
6. Rinse in 3 changes of distilled water 6 dip each.
7. Dip slides in 0.02% alcoholic Fast Green for 30 seconds.
8. Dip slides in fresh 1% acetic acid solution 8 dips.
9. Rinse quickly in distilled water 6 dips.
10. Quickly dip slides in 95% ethanol 6 dips.
11. Dehydrate in 2 changes of 100% ethanol 8 dips each.
12. Clear in two or three changes of Xylene for 2minute each.
13. Mount with DPX and Cover Slides.

Results: Articular cartilage is stained red because of its proteoglycan content. Subchondral bone stained green and calcified cartilage in between stained light pink

E. Alizarin Red staining solution:

Alizarin Red S (Sigma-Aldrich) 2g in 100 deionized water mixed well and PH was set to 4.2 by a pH meter using 0.1N NaOH and 10% Acetic acid. The dye was filtered before use.

Protocol:

1. Washing the cells with warm 1XPBS.
2. Fix the cells with formalin 10% at room temperature.
3. Remove the fixative and wash the cells with PBS.
4. Add filtered AlizarinRed (2-5 minutes at room temperature).
5. Rinse the cells with PBS one to two times follow with 4 times dw.
6. Counterstain the nucleus with Hematoxylin.

7. Dehydrate and clear with Acetone 100%, Acetone/Xylene 50/50 Xylene 100% 1min each
8. Mount the slides with DPX and Cover Slides.
9. Capture images.

F. Oil Red staining for cell culture:

Oil Red stock solution: Sigma (Cat# 0-0625)

0.35g Oil Red in 100ml Isopropanol, Stir, Filter store at RT up to one year

Oil Red working solution: Mix 3 parts of Oil Red O stock with two parts of dw (stable for 2 hours)

Procedure:

1. Wash the cells with warm 1XPBS.
2. Fix the cells with 10% formalin (20 minutes RT).
3. Wash with dw twice.
4. Rinse with 60% Isopropanol.
5. Stain with fresh Oil Red working solution (15 minutes).
6. Rinse with dw.
7. Stain with Hematoxylin 1 minute.
8. Rinse with dw (4 times).
9. Capture images.

APPENDIX C: Reagents preparation and protocol for TEM

A. Toluidine Blue staining (Alkaline Toluidine Blue) for semi thin sections:

Solution A: Toluidine Blue 1g, 1% Borax Solution in distilled water 100ml

Solution B: Pyronin G (EM Grade) 1g, 1% Borax solution in distilled water 100ml

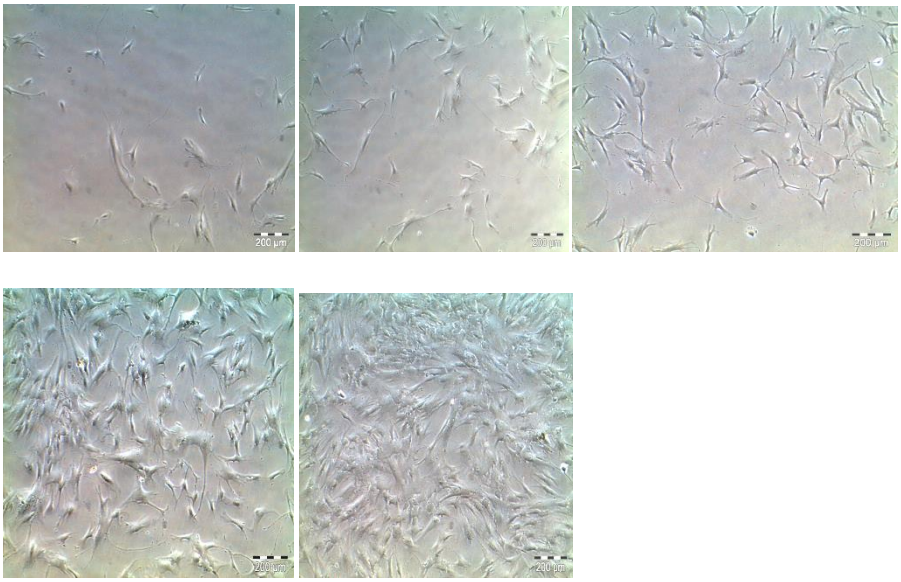
Mix 4 parts of Solution A to one part of solution B. Keep in amber bottles at room temperature. Filter before use.

B. Uranyl acetate Lead citrate staining:

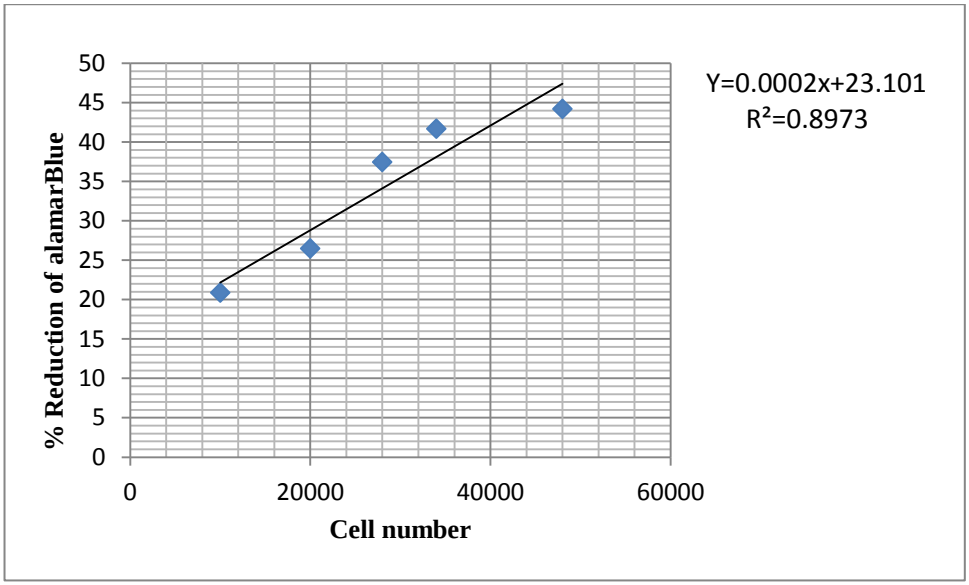
Semi thin sections collected on copper grids, and stained with Uranyl Acetate & Lead Citrate.

1. Centrifuge the stain solutions of Uranyl Acetate and Lead Citrate at 3000 rpm for 10 minutes to sediment the possible crystals in the stains. Then filter (0.22 μm) the solutions prior use.
2. Put drops of Uranyl acetate solution on top of a dental wax in a Petri dish and float the grids upside down (the sample faced the stain) for 10 minutes.
3. Wash in filtered ddw three times each time 10 dips and dry with a small piece of a filter paper. (Care must be taken not to damage the sample when handling with fine forceps).
4. Stain with lead citrate and wash with filtered ddw the same way as described for Uranyl Acetate (put some pellets of NaOH in the Petri dish to absorb the humidity).
5. Place the grids in a clean Petri dish on a piece of filter paper in desiccators until viewing with TEM.

APPENDIX D: AlamarBlue Standard Curve and formula



A-F Human MSCs in ascending densities of 10000,20000, 30000, 35000, 50000 , 48 hours after seeding in 6 well plate, growth medium, Phase contrast microscopy 4X.



AlamarBlue standard curve after 3 hour incubation of AlamarBlue 10% with MSCs.

AlamarBlue equation

$$Percentage\ reduction = \frac{(O2xA1) - (O1XA2)}{(R1XN2) - (R2XN1)} X100$$

O1 = molar extinction coefficient (E) of oxidized AlamarBlue (Blue) at 570nm

O2 = E of oxidized AlamarBlue at 600nm

R1 = E of reduced AlamarBlue (Red) at 570nm

R2 = E of reduced AlamarBlue at 600nm

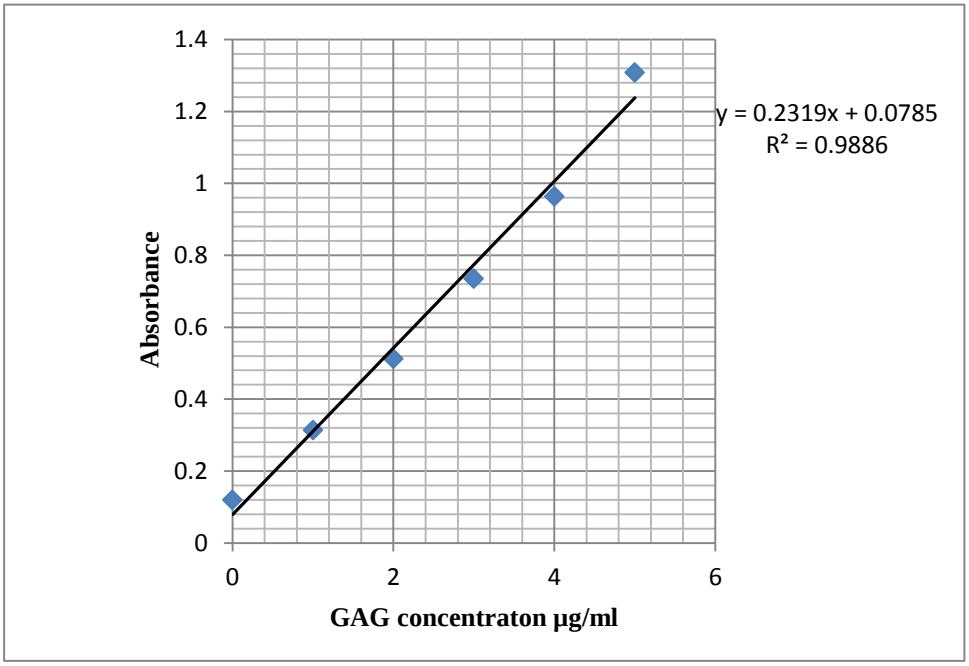
A1 = absorbance of test wells at 570nm

A2 = absorbance of test wells at 600nm

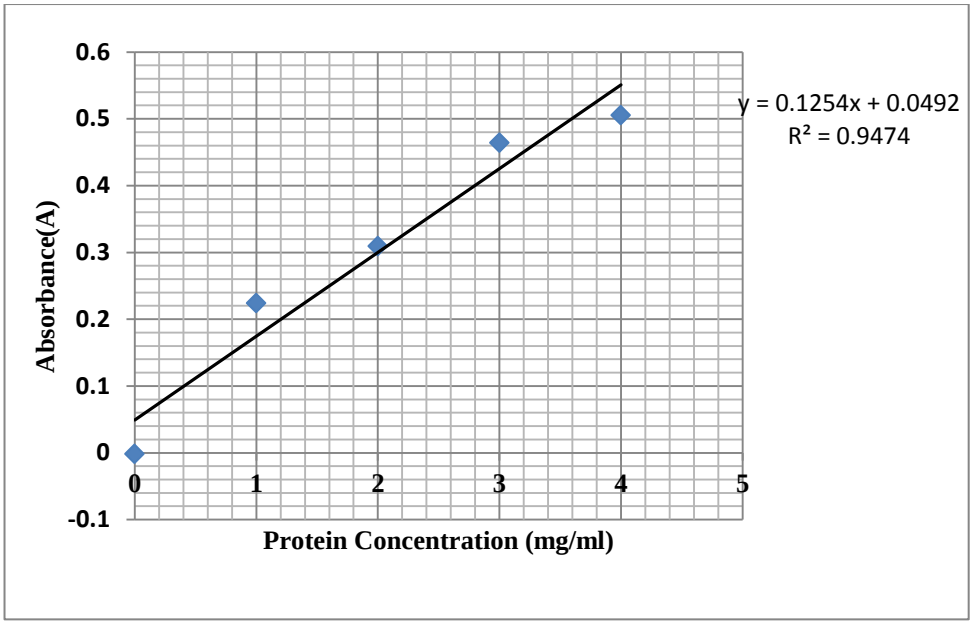
N1 = absorbance of negative control well (media plus AlamarBlue but no cells) at 570nm

N2 = absorbance of negative control well (media plus AlamarBlue but no cells) at 600nm

APENDIX E: GAG and Protein assay standard curve



A. Sample standard curve for GAG analysis.



B. Sample standard curve for protein analysis.

APPENDIX F: Statistical analysis for gene expression

Kruskal-Wallis test

Ranks

	Ser	N	Mean Rank	P-Value
D3 Aggrecan expression	Alginate	3	8.00	0.027
	Pellet	3	5.00	
	Monolayer	3	2.00	
	Total	9		
D12 Aggrecan expression	Alginate	3	8.00	0.039
	Pellet	3	4.67	
	Monolayer	3	2.33	
	Total	9		
D21 Aggrecan expression	Alginate	3	8.00	0.027
	Pellet	3	5.00	
	Monolayer	3	2.00	
	Total	9		

Ranks

Time points		N	Mean Rank	P-Value
Alginate Aggrecan	D3	3	2.00	
	D12	3	5.33	0.039
	D21	3	7.67	
	Total	9		
Pellet Aggrecan	D3	3	3.83	
	D12	3	3.17	0.063
	D21	3	8.00	
	Total	9		
Monolayer Aggrecan	D3	3	2.83	0.172
	D12	3	5.17	
	D21	3	7.00	
	Total	9		

Ranks

	Ser	N	Mean Rank	P-Value
D3 Sox9 expression	Alginate	3	6.00	
	Pellet	3	3.33	0.427
	Monolayer	3	5.67	
	Total	9		
D12 Sox9 expression	Alginate	3	8.00	0.05
	Pellet	3	3.00	
	Monolayer	3	4.00	
	Total	9		
D21 Sox9 expression	Alginate	3	8.00	0.05
	Pellet	3	3.67	
	Monolayer	3	3.33	
	Total	9		

Ranks

	Time points	N	Mean Rank	P-Value
Alginate Sox9	D3	3	2.00	
	D12	3	5.67	0.05
	D21	3	7.33	
	Total	9		
Pellet Sox9	D3	3	3.67	0.288
	D12	3	4.33	
	D21	3	7.00	
	Total	9		
Monolayer Sox9	D3	3	3.00	0.276
	D12	3	6.33	
	D21	3	5.67	
	Total	9		

Ranks

	Ser	N	Mean Rank	P-Value
D3 Col X expression	Algiante	3	5.33	
	Pellet	3	2.00	0.039
	Monolayer	3	7.67	
	Total	9		
D12 Col X expression	Algiante	3	4.00	
	Pellet	3	3.00	
	Monolayer	3	8.00	0.061
	Total	9		
D21 Col X expression	Algiante	3	2.00	
	Pellet	3	7.00	0.059
	Monolayer	3	6.00	
	Total	9		

Ranks

	Time points	N	Mean Rank	p-Value
Col X expression in Alginate	Day 3	3	8.00	
	Day 12	3	2.33	
	Day 21	3	4.67	0.039
	Total	9		
Col X expression in Pellet	Day 3	3	3.67	0.066
	Day 12	3	3.33	
	Day 21	3	8.00	
	Total	9		
Col X expression in Monolayer	Day 3	3	3.83	0.084
	Day 12	3	7.83	
	Day 21	3	3.33	
	Total	9		

Ranks

	Ser	N	Mean Rank	P-Value
Day 3 Col I expression	Alginate	3	5.00	
	Pellet	3	2.00	0.027
	Monolayer	3	8.00	
	Total	9		
Day 12 Col I expression	Alginate	3	7.67	
	Pellet	3	2.00	
	Monolayer	3	5.33	0.039
	Total	9		
Day 21 Col I expression	Alginate	3	7.83	
	Pellet	3	2.00	
	Monolayer	3	5.17	0.032
	Total	9		

Ranks

	Time points	N	Mean Rank	P-Value
Alginate Col I	D3	3	2.00	
	D12	3	7.00	0.061
	D21	3	6.00	
	Total	9		
Pellet Col I	D3	3	2.33	
	D12	3	4.67	0.039
	D21	3	8.00	
	Total	9		
Monolayer Col I	D3	3	2.17	
	D12	3	6.17	
	D21	3	6.67	0.086
	Total	9		

Ranks

	Ser	N	Mean Rank	P-Value
Day 3 Col II gene expression	Alginate	3	8.00	
	Pellet	3	3.00	
	Monolayer	3	4.00	0.05
	Total	9		
Day 12 Col II gene expression	Alginate	3	8.00	
	Pellet	3	5.00	
	Monolayer	3	2.00	0.027
	Total	9		
Day 21 Col II gene expression	Alginate	3	8.00	
	Pellet	3	5.00	0.027
	Monolayer	3	2.00	
	Total	9		

Ranks

	Time point	N	Mean Rank	P-Value
Alginate Col II	Day 3	3	2.00	
	Day 12	3	5.33	
	Day 21	3	7.67	0.039
	Total	9		
Pellet Col II	Day 3	3	2.00	
	Day 12	3	5.00	0.027
	Day 21	3	8.00	
	Total	9		
Monolayer Col II	Day 3	3	2.00	
	Day 12	3	7.00	0.059
	Day 21	3	6.00	
	Total	9		

Ranks

	Ser	N	Mean Rank	P-Value
Day 3 RunX2	Alginate	3	6.00	
	Pellet	3	2.00	0.061
	Monolayer	3	7.00	
	Total	9		
Day 12 RunX2	Alginate	3	4.00	
	Pellet	3	3.00	
	Monolayer	3	8.00	0.061
	Total	9		
Day 21 RunX2	Alginate	3	2.00	
	Pellet	3	8.00	0.027
	Monolayer	3	5.00	
	Total	9		

Ranks

Time point		N	Mean Rank	P-Value
Alginate RunX2	Day 3	3	8.00	
	Day 12	3	4.00	0.061
	Day 21	3	3.00	
	Total	9		
Pellet RunX2	Day 3	3	4.00	0.061
	Day 12	3	3.00	
	Day 21	3	8.00	
	Total	9		
Monolayer RunX2	Day 3	3	4.50	
	Day 12	3	7.50	0.120
	Day 21	3	3.00	
	Total	9		

Ranks

	ser	N	Mean Rank	P-Value
Day3 NCAM1expression	Alginate	3	4.33	
	Pellet	3	2.67	0.05
	Monolayer	3	8.00	
	Total	9		
Day 12 NCAM1 expression	Alginate	3	5.00	0.027
	Pellet	3	2.00	
	Monolayer	3	8.00	
	Total	9		
Day 21 NCAM1 expression	Alginate	3	8.00	0.055
	Pellet	3	4.17	
	Monolayer	3	2.83	
	Total	9		

Ranks

	Time point	N	Mean Rank	P-Vallue
Alginate NCAM1	Day3	3	2.00	0.05
	Day 12	3	5.67	
	Day 21	3	7.33	
	Total	9		
Pellet NCAM1	Day3	3	3.67	0.193
	Day 12	3	4.00	
	Day 21	3	7.33	
	Total	9		
Monolayer NCAM1	Day3	3	5.50	0.027
	Day 12	3	7.50	
	Day 21	3	2.00	
	Total	9		

Ranks

	Ser	N	Mean Rank	P-Value
Day3 NCad	Alginate	3	3.67	
	Pellet	3	3.33	
	Monolayer	3	8.00	0.066
	Total	9		
Day 12 NCad	Alginate	3	4.67	
	Pellet	3	2.67	
	Monolayer	3	7.67	0.079
	Total	9		
Day 21 NCad	Alginate	3	5.83	
	Pellet	3	6.17	
	Monolayer	3	3.00	0.295
	Total	9		

Ranks

	Time point	N	Mean Rank	P-Value
Alginate NCad	Day 3	3	2.00	
	Day 12	3	6.00	
	Day 21	3	7.00	0.061
	Total	9		
Pellet NCad	Day 3	3	2.67	
	Day 12	3	4.33	0.051
	Day 21	3	8.00	
	Total	9		
Monolayer NCad	Day 3	3	3.50	
	Day 12	3	6.50	
	Day 21	3	5.00	0.404
	Total	9		

Mann-Whitney U test:

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
Day3 NCad	Alginate	3	3.67	11.00	
	Pellet	3	3.33	10.00	0.827
	Total	6			
Day 12 NCad	Alginate	3	4.33	13.00	0.275
	Pellet	3	2.67	8.00	
	Total	6			
Day 21 NCad	Alginate	3	3.83	11.50	0.658
	Pellet	3	3.17	9.50	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day3 NCad expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 NCad expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 21 NCad expression	Pellet	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
Day3 NCad	Alginate	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 NCad	Alginate	3	2.33	7.00	0.127
	Monolayer	3	4.67	14.00	
	Total	6			
Day 21 NCad	Alginate	3	4.00	12.00	0.513
	Monolayer	3	3.00	9.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate NCad	Day 3	3	2.00	6.00	0.05
	Day 12	3	5.00	15.00	
	Total	6			
Pellet NCad	Day 3	3	2.67	8.00	0.275
	Day 12	3	4.33	13.00	
	Total	6			
Monolayer NCad	Day 3	3	2.50	7.50	0.184
	Day 12	3	4.50	13.50	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate NCad	Day 12	3	3.00	9.00	0.513
	Day 21	3	4.00	12.00	
	Total	6			
Pellet NCad	Day 12	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Monolayer NCad	Day 12	3	4.00	12.00	0.513
	Day 21	3	3.00	9.00	
	Total	6			

anks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate NCad	Day 3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Pellet NCad	Day 3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Monolayer NCad	Day 3	3	3.00	9.00	0.513
	Day 21	3	4.00	12.00	
	Total	6			

Ranks

	ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 NCAM1	Alginate	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 NCAM1	Alginate	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 21 NCAM1	Alginate	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Ranks

	ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 NCAM1	Alginate	3	4.33	13.00	0.275
	Pellet	3	2.67	8.00	
	Total	6			
Day 12 NCAM1	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
Day 21 NCAM1 expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			

Ranks

	ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 NCAM1	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 NCAM1	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 21 NCAM1	Pellet	3	4.17	12.50	0.376
	Monolayer	3	2.83	8.50	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate NCAM1	Day3	3	2.00	6.00	0.05
	Day 12	3	5.00	15.00	
	Total	6			
Pellet NCAM1	Day3	3	3.33	10.00	0.827
	Day 12	3	3.67	11.00	
	Total	6			
Monolayer NCAM1	Day3	3	2.00	6.00	0.05
	Day 12	3	5.00	15.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate NCAM1	Day 12	3	2.67	8.00	0.275
	Day 21	3	4.33	13.00	
	Total	6			
Pellet NCAM1	Day 12	3	2.33	7.00	0.127
	Day 21	3	4.67	14.00	
	Total	6			
Monolayer NCAM1	Day 12	3	5.00	15.00	0.05
	Day 21	3	2.00	6.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate NCAM1	Day3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Pellet NCAM1	Day3	3	2.33	7.00	0.127
	Day 21	3	4.67	14.00	
	Total	6			
Monolayer NCAM1	Day3	3	5.00	15.00	0.05
	Day 21	3	2.00	6.00	
	Total	6			

Ranks Aggrecan Mann-Whittny

Sig.(2-tailed)

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Aggrecan _	D3	3	2.00	6.00	
	D21	3	5.00	15.00	0.05
	Total	6			
Pellet Aggrecan _	D3	3	2.00	6.00	
	D21	3	5.00	15.00	0.05
	Total	6			
Monolayer Aggrecan _	D3	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			

anks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Aggrecan _	D3	3	2.00	6.00	
	D12	3	5.00	15.00	0.05
	Total	6			
Pellet Aggrecan _	D3	3	3.83	11.50	0.658
	D12	3	3.17	9.50	
	Total	6			
Monolayer Aggrecan _	D3	3	2.83	8.50	0.376
	D12	3	4.17	12.50	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Aggrecan _	D12	3	2.33	7.00	0.127
	D21	3	4.67	14.00	
	Total	6			
Pellet Aggrecan _	D12	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			
Monolayer Aggrecan _	D12	3	3.00	9.00	0.513
	D21	3	4.00	12.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
D3 Aggrecan	Alginate	3	5.00	15.00	
	Pellet	3	2.00	6.00	0.05
	Total	6			
D12 Aggrecan	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
D21 Aggrecan	Alginate	3	5.00	15.00	
	Pellet	3	2.00	6.00	0.05
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
D3 Aggrecan	Pellet	3	5.00	15.00	
	Monolayer	3	2.00	6.00	0.05
	Total	6			
D12 Aggrecan	Pellet	3	4.67	14.00	
	Monolayer	3	2.33	7.00	0.127
	Total	6			
D21 Aggrecan	Pellet	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
D3 Aggrecan	Alginate	3	5.00	15.00	
	Monolayer	3	2.00	6.00	0.05
	Total	6			
D12 Aggrecan	Alginate	3	5.00	15.00	
	Monolayer	3	2.00	6.00	0.05
	Total	6			
D21 Aggrecan	Alginate	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Sig.(2-tailed)

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Sox9	D3	3	2.00	6.00	0.05
	D12	3	5.00	15.00	
	Total	6			
Pellet Sox9	D3	3	3.67	11.00	0.827
	D12	3	3.33	10.00	
	Total	6			
Monolayer Sox9	D3	3	2.67	8.00	0.246
	D12	3	4.33	13.00	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Sox9	D12	3	2.67	8.00	
	D21	3	4.33	13.00	0.275
	Total	6			
Pellet Sox9	D12	3	3.00	9.00	
	D21	3	4.00	12.00	0.513
	Total	6			
Monolayer Sox	D12	3	4.00	12.00	0.513
	D21	3	3.00	9.00	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Sox9	D3	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			
Pellet Sox9	D3	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			
Monolayer Sox9	D3	3	2.33	7.00	0.121
	D21	3	4.67	14.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
D3 Sox9 expression	Alginate	3	4.33	13.00	0.275
	Pellet	3	2.67	8.00	
	Total	6			
D12 Sox9 expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
D21 Sox9 expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
D3 Sox9 expression	Alginate	3	3.67	11.00	0.825
	Monolayer	3	3.33	10.00	
	Total	6			
D12 Sox9 expression	Alginate	3	5.00	15.00	
	Monolayer	3	2.00	6.00	0.05
	Total	6			
D21 Sox9 expression	Alginate	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
D3 Sox9 expression	Pellet	3	2.67	8.00	
	Monolayer	3	4.33	13.00	0.268
	Total	6			
D12 Sox9 expression	Pellet	3	3.00	9.00	0.513
	Monolayer	3	4.00	12.00	
	Total	6			
D21 Sox9 expression	Pellet	3	3.67	11.00	0.827
	Monolayer	3	3.33	10.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 Col I expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
Day 12 Col I expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
Day 21 Col I expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 Col I expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 Col I expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 21 Col I expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 Col I expression	Alginate	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 Col I expression	Alginate	3	4.67	14.00	0.127
	Monolayer	3	2.33	7.00	
	Total	6			
Day 21 Col I expression	Alginate	3	4.83	14.50	0.077
	Monolayer	3	2.17	6.50	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Col I	D3	3	2.00	6.00	0.05
	D12	3	5.00	15.00	
	Total	6			
Pellet Col I	D3	3	2.33	7.00	0.127
	D12	3	4.67	14.00	
	Total	6			
Monolayer Col I	D3	3	2.17	6.50	0.077
	D12	3	4.83	14.50	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Col I	D12	3	4.00	12.00	0.513
	D21	3	3.00	9.00	
	Total	6			
Pellet Col I	D12	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			
Monolayer Col I	D12	3	3.33	10.00	0.827
	D21	3	3.67	11.00	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Alginate Col I	D3	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			
Pellet Col I	D3	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			
Monolayer Col I	D3	3	2.00	6.00	0.05
	D21	3	5.00	15.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 Col II gene expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
Day 12 Col II gene expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
Day 21 Col II gene expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 Col II gene expression	Alginate	3	5.00	15.00	
	Monolayer	3	2.00	6.00	0.046
	Total	6			
Day 12 Col II gene expression	Alginate	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			
Day 21 Col II gene expression	Alginate	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 Col II gene expression	Pellet	3	3.00	9.00	
	Monolayer	3	4.00	12.00	0.507
	Total	6			
Day 12 Col II gene expression	Pellet	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			
Day 21 Col II gene expression	Pellet	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	p-Value
Alginate Col II	Day 3	3	2.00	6.00	
	Day 12	3	5.00	15.00	0.05
	Total	6			
Pellet Col II	Day 3	3	2.00	6.00	
	Day 12	3	5.00	15.00	0.05
	Total	6			
Monolayer Col II	Day 3	3	2.00	6.00	0.046
	Day 12	3	5.00	15.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	p-Value
Alginate Bead Col II	Day 12	3	2.33	7.00	0.125
	Day 21	3	4.67	14.00	
	Total	6			
Pellet Col II	Day 12	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Monolaye colll	Day 12	3	4.00	12.00	0.513
	Day 21	3	3.00	9.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate Col II	Day 3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Pellet Col II	Day 3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Monolayer Col II	Day 3	3	2.00	6.00	0.046
	Day 21	3	5.00	15.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 RunX2 expression	Alginate	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
Day 12 RunX2 expression	Alginate	3	4.00	12.00	0.513
	Pellet	3	3.00	9.00	
	Total	6			
Day 21 RunX2 expression	Alginate	3	2.00	6.00	0.05
	Pellet	3	5.00	15.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 RunX2 expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 12 RunX2 expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 21 RunX2 expression	Pellet	3	5.00	15.00	0.05
	Monolayer	3	2.00	6.00	
	Total	6			

nks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
Day 3 RunX2 expression	Alginate	3	3.00	9.00	0.513
	Monolayer	3	4.00	12.00	
	Total	6			
Day 12 RunX2 expression	Alginate	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
Day 21 RunX2 expression	Alginate	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate RunX2	Day 3	3	5.00	15.00	0.05
	Day 12	3	2.00	6.00	
	Total	6			
Monolayer	Day 3	3	2.50	7.50	0.184
	Day 12	3	4.50	13.50	
	Total	6			
Pellet	Day 3	3	4.00	12.00	0.513
	Day 12	3	3.00	9.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate Runx2	Day 12	3	4.00	12.00	0.513
	Day 21	3	3.00	9.00	
	Total	6			
Monolayer	Day 12	3	5.00	15.00	0.05
	Day 21	3	2.00	6.00	
	Total	6			
Pellet	Day 12	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			

Ranks

	Time point	N	Mean Rank	Sum of Ranks	P-Value
Alginate Run X2	Day 3	3	5.00	15.00	0.05
	Day 21	3	2.00	6.00	
	Total	6			
Monolayer	Day 3	3	4.00	12.00	0.513
	Day 21	3	3.00	9.00	
	Total	6			
Pellet	Day 3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
D3 Col X expression	Algiante	3	5.00	15.00	0.05
	Pellet	3	2.00	6.00	
	Total	6			
D12 Col X expression	Algiante	3	4.00	12.00	0.513
	Pellet	3	3.00	9.00	
	Total	6			
D21 Col X expression	Algiante	3	2.00	6.00	0.05
	Pellet	3	5.00	15.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	P-Value
D3 Col X expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
D12 Col X expression	Pellet	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
D21 Col X expression	Pellet	3	4.67	14.00	0.121
	Monolayer	3	2.33	7.00	
	Total	6			

Ranks

	Ser	N	Mean Rank	Sum of Ranks	p-Value
D3 Col X expression	Algiante	3	2.33	7.00	0.127
	Monolayer	3	4.67	14.00	
	Total	6			
D12 Col X expression	Algiante	3	2.00	6.00	0.05
	Monolayer	3	5.00	15.00	
	Total	6			
D21 Col X expression	Algiante	3	2.00	6.00	0.046
	Monolayer	3	5.00	15.00	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Col X expression in Alginate	Day 3	3	5.00	15.00	0.05
	Day 12	3	2.00	6.00	
	Total	6			
Col X expression in Pellet	Day 3	3	3.67	11.00	0.827
	Day 12	3	3.33	10.00	
	Total	6			
Col X expression in Monolayer	Day 3	3	2.17	6.50	0.077
	Day 12	3	4.83	14.50	
	Total	6			

Ranks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Col X expression in Alginate	Day 12	3	2.33	7.00	0.127
	Day 21	3	4.67	14.00	
	Total	6			
Col X expression in Pellet	Day 12	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Col X expression in Monolayer	Day 12	3	5.00	15.00	0.046
	Day 21	3	2.00	6.00	
	Total	6			

anks

	Time points	N	Mean Rank	Sum of Ranks	P-Value
Col X expression in Alginate	Day 3	3	5.00	15.00	0.05
	Day 21	3	2.00	6.00	
	Total	6			
Col X expression in Pellet	Day 3	3	2.00	6.00	0.05
	Day 21	3	5.00	15.00	
	Total	6			
Col X expression in Monolayer	Day 3	3	3.67	11.00	0.827
	Day 21	3	3.33	10.00	
	Total	6			

APPENDIX G: Macroscopic Evaluation of the repaired cartilage

Brittberg Scoring System

Graft Assesment Maximal Score=12*	Criteria	Points
Degree of defect repair		4
	in level with surrounding cartilage	3
	75% repair of defect depth	2
	50% repair of defect depth	1
	25% repair of defect depth	0
	0% repair of defect depth	
Integration to border zone	Complete integration with surrounding cartilage	4
	Demarcating border <1mm ¾ of graft integrated, ¼ with a notable border>1mm width	3
	½ of graft integrated with surrounding cartilage, ½	2
	With a notable border>1mm	1
	Form no contact to ¼ of graft integrated with	0
	surrounding cartilage	
Macroscopic appearance	Intact smooth surface	4
	Fibrillated surface	3
	Small scattered fissures or cracks	2
	Several small or few but large fissures	1
	Total degeneration of grafted area	0

*For a classification as a biologically acceptable appearance a minimal score of 7 points with at least 3 in Group 1(degree of defect repair) and 2 in the other groups are necessary.

APPENDICX H: Microscopic evaluation of repaired cartilage

O'Driscoll scoring system

O'Driscoll Histological Scoring System

Characteristics	Score
Nature of predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0
Safranin O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures 25 to 100 percent of thickness	1
Severe disrupting including fibrillation	0
Structural integrity	
Normal	2
Slight disruption including cysts	1
Severe disintegration	0
Thickness	
100 percent of normal adjacent cartilage	2
50 to 100 percent of normal cartilage	1

0 to 50 percent of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end and partially at both ends	1
Not bonded	0
Freedom from cellular changes of degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellulartiy	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
<25 percent of the cells	1
25 to 100 percent of the cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0
Total Score	

Appendix I : Raw data for Brittberg and O'Driscoll scoring

N=6 in each group	O'Driscoll scores	
	Left knee	Right knee
Alginate	7	6.4
Alginate-MSC	8.166666667	11.66666667
Alginat-CMSC	6.7	11

N=6 in each group	Brittberg Score	
	Left knee	Right knee
Alginate	4.8	5.6
Alginate-MSC	5.5	7.9
Alginat-CMSC	6	9.5

APPENDIX J: Flow cytometry analysis of MSCs CD markers

Tube: Unstained

Population	#Events	%Parent	%Total
All Events	13,982	####	100.0
MSC	11,794	84.4	84.4
Q1	0	0.0	0.0
Q2	0	0.0	0.0
Q3	11,794	100.0	84.4
Q4	0	0.0	0.0
Q1-1	0	0.0	0.0
Q2-1	1	0.0	0.0
Q3-1	11,793	100.0	84.3
Q4-1	0	0.0	0.0
Q1-2	9	0.1	0.1
Q2-2	1	0.0	0.0
Q3-2	11,773	99.8	84.2
Q4-2	11	0.1	0.1
Q1-3	0	0.0	0.0
Q2-3	0	0.0	0.0
Q3-3	11,669	98.9	83.5
Q4-3	125	1.1	0.9
Q1-4	0	0.0	0.0
Q2-4	0	0.0	0.0
Q3-4	11,794	100.0	84.4
Q4-4	0	0.0	0.0
Q1-5	2	0.0	0.0
Q2-5	0	0.0	0.0
Q3-5	11,792	100.0	84.3
Q4-5	0	0.0	0.0
Q1-6	0	0.0	0.0
Q2-6	0	0.0	0.0
Q3-6	11,793	100.0	84.3
Q4-6	1	0.0	0.0
Q1-7	1	0.0	0.0
Q2-7	0	0.0	0.0
Q3-7	11,792	100.0	84.3
Q4-7	1	0.0	0.0
Q1-8	5	0.0	0.0
Q2-8	8	0.1	0.1
Q3-8	11,681	99.0	83.5
Q4-8	100	0.8	0.7
Q1-9	0	0.0	0.0
Q2-9	1	0.0	0.0
Q3-9	11,215	95.1	80.2
Q4-9	578	4.9	4.1
Q1-10	0	0.0	0.0
Q2-10	0	0.0	0.0
Q3-10	10,816	91.7	77.4
Q4-10	978	8.3	7.0
Q1-11	0	0.0	0.0
Q2-11	1	0.0	0.0
Q3-11	11,609	98.4	83.0
Q4-11	184	1.6	1.3
Q1-12	1	0.0	0.0
Q2-12	0	0.0	0.0
Q3-12	11,441	97.0	81.8
Q4-12	352	3.0	2.5

Tube: CD73/CD34/CD105/CD44/CD90/CD45

Population	#Events	%Parent	%Total
All Events	13,674	####	100.0
MSC	11,004	80.5	80.5
Q1	0	0.0	0.0
Q2	6	0.1	0.0
Q3	623	5.7	4.6
Q4	10,375	94.3	75.9
Q1-1	233	2.1	1.7
Q2-1	10,764	97.8	78.7
Q3-1	2	0.0	0.0
Q4-1	5	0.0	0.0
Q1-2	22	0.2	0.2
Q2-2	10,982	99.8	80.3
Q3-2	0	0.0	0.0
Q4-2	0	0.0	0.0
Q1-3	0	0.0	0.0
Q2-3	11,004	100.0	80.5
Q3-3	0	0.0	0.0
Q4-3	0	0.0	0.0
Q1-4	10,920	99.2	79.9
Q2-4	57	0.5	0.4
Q3-4	27	0.2	0.2
Q4-4	0	0.0	0.0
Q1-5	10,994	99.9	80.4
Q2-5	5	0.0	0.0
Q3-5	5	0.0	0.0
Q4-5	0	0.0	0.0
Q1-6	10,858	98.7	79.4
Q2-6	145	1.3	1.1
Q3-6	1	0.0	0.0
Q4-6	0	0.0	0.0
Q1-7	12	0.1	0.1
Q2-7	3	0.0	0.0
Q3-7	10,802	98.2	79.0
Q4-7	187	1.7	1.4
Q1-8	1	0.0	0.0
Q2-8	11,003	100.0	80.5
Q3-8	0	0.0	0.0
Q4-8	0	0.0	0.0
Q1-9	0	0.0	0.0
Q2-9	15	0.1	0.1
Q3-9	1	0.0	0.0
Q4-9	10,988	99.9	80.4
Q1-10	1	0.0	0.0
Q2-10	11,002	100.0	80.5
Q3-10	0	0.0	0.0
Q4-10	1	0.0	0.0
Q1-11	0	0.0	0.0
Q2-11	15	0.1	0.1
Q3-11	0	0.0	0.0
Q4-11	10,989	99.9	80.4
Q1-12	0	0.0	0.0
Q2-12	15	0.1	0.1
Q3-12	0	0.0	0.0
Q4-12	10,989	99.9	80.4

LIST OF PUBLICATIONS

1. **Dashtdar H**., Murali M.R., Azlina Amir Abbas, Suhaeb A.M., Selvaratnam L., Tay L.C., & Tunku Kamarul (2013). "PVA-chitosan composite hydrogel versus alginate beads as a potential mesenchymal stem cell carrier for the treatment of focal cartilage defects." Knee Surg Sports Traumatol Arthrosc.

2. **Dashtdar H**., Rothan H.A., Tay T., Raja Elina Ahmad , Razif Ali, Tay L.X., Chong P.P., & Tunku Kamarul (2011). "A Preliminary Study Comparing the Use of Allogenic Chondrogenic Pre-Differentiated and Undifferentiated Mesenchymal Stem Cells for the Repair of Full Thickness Articular Cartilage Defects in Rabbits." Journal of Orthopaedic Research 29(9): 1336-1342.

3. Tay L.X., Raja Elina Ahmad, **Dashtdar H**., Tay K.W, Masjuddin T., Ab-Rahim S., Chong P.P., Selvaratnam L., & Tunku Kamarul (2012). "Treatment outcomes of alginate-embedded allogenic mesenchymal stem cells versus autologous chondrocytes for the repair of focal articular cartialge defects in a rabbit model." American Journal of Sports Medicine 40(1): 83-90.

4. **Havva Dashtdar**, Malliga Raman Murali, Lakshmi Selvaratnam, Hanumantha Rao Balaji Raghavendran, Tunku Sara Ahmad, Tunku Kamarul (2014). Comparative analysis of chondrogenic, hypertrophic and cell adhesion molecules expression and ultrastructural changes of human bone marrow derived mesenchymal stromal cells during chondrogenic differentiation in alginate bead, pellet and monolayer culture. (In preparation)